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The Development of BSN272 From Prevention of Diet-Induced Hyperlipidemia in Mice to a Potential Therapy For Prader-Willi Syndrome in Humans

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THE DEVELOPMENT OF BSN272 FROM PREVENTION OF DIET-INDUCED HYPERLIPIDEMIA IN MICE TO A POTENTIAL THERAPY FOR PRADER-WILLI SYNDROME IN HUMANS

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Pharmacy
at the University of Kentucky

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ABSTRACT OF DISSERTATION

THE DEVELOPMENT OF BSN272 FROM PREVENTION OF DIET-INDUCED HYPERLIPIDEMIA IN MICE TO A POTENTIAL THERAPY FOR PRADER-WILLI SYNDROME IN HUMANS

While type 2 diabetes mellitus (T2DM) is distinguished as a disorder of blood glucose homeostasis, the vast majority of patients afflicted with this disease in the US present with the entire complement of the metabolic syndrome: abdominal obesity, hypertriglyceridemia, low HDL levels, elevated blood pressure, and elevated fasting plasma glucose. Current guidelines aim to treat each of these disorders, but do so individually even though they are manifestations of common pathologies. In other words, a patient will be prescribed different medications for diabetes, blood pressure, CVD prevention, etc., while the only single treatment aimed at the myriad of disorders together is changes to dietary and exercise habits. Diet and exercise, when executed properly, has been shown consistently to be the most effective treatment for T2DM. The rate of type 2 diabetes in the US is close to 10%, but the rate of type 2 diabetes is even higher in patients with Prader-Willi Syndrome (a genetic disease that causes excessive weight gain from overeating, resulting in approximately double the risk of diabetes). BSN272 is a combination of 2 molecules (D-tagatose and trans-polydatin) that have shown benefits in treating individual manifestations of the metabolic syndrome presentation as monotherapy and have proved synergistic when taken together to treat the entire complement of the disorders.

Previous research in our lab with D-tagatose included safety studies in animal models and humans for use as a dietary ingredient which culminated with the generally recognized as safe (GRAS) designation by the FDA and EU for use in foods. Based on these exposures to determine safety, an IND was granted to evaluate the safety and efficacy of D-tagatose in treating hyperglycemia in T2DM. Phase 2 and 3 global clinical trials were completed showing D-tagatose to be highly safe and moderately effective. The decision was made to search for a molecular complement to D-tagatose that could more completely treat T2DM.

Dihydromyricetin and trans-polydatin were identified through literature searches as potentially synergistic with D-tagatose. Both molecules were tested in combination with D-tagatose for their ability to prevent diet-induced elevations in cholesterol markers in ApoE$^{-/-}$ mice. Dihydromyricetin appeared to be additively effective with D-tagatose for lipids, but trans-polydatin showed synergy in prevention of cholesterol elevations as results were greater for the combination than were the additive benefits of either as monotherapy. Trans-polydatin also showed this property in a hyperlipidemic hamster model and a LDLr$^{-/-}$ mouse model.

Following safety and efficacy results in three animal models for BSN272, an exploratory Phase 1 PK microdose study was submitted to the FDA in an IND filing to investigate whether the presence of D-tagatose has any effects on the kinetics of trans-polydatin when administered in concert compared to trans-polydatin alone. The indication sought for the IND is Prader-Willi Syndrome (PWS) due to the added potential of D-tagatose to impart satiety and reduce weight gain. PWS is a genetic disorder in which patients develop insatiable hunger that leads to obesity and often diabetes while in childhood. There is currently only symptomatic treatment of the disease and none of the medications in treatment guidelines for adult type 2 diabetes are approved for pediatric use.

KEYWORDS: Polydatin, piceid, D-tagatose, tagatose, type 2 diabetes, Prader-Willi syndrome

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12/14/16

Date

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12/14/16

To my family for their continued support

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Table of Contents

List of Tables

Chapter 3 – BSN723T Prevents Atherosclerosis and Weight Gain in ApoE Knockout Mice Fed a Western Diet

Chapter 4 – BSN272 Prevents Western Diet-Induced Atherosclerosis and Excess Weight Gain in ApoE$^{-/-}$ Mice

List of Figures

Chapter 1 – Introduction

This chapter provides an introduction to the concepts and previous studies conducted that are pertinent to understanding the chapters within this dissertation. The dissertation is made up primarily of published articles. Where the introduction and preface to each chapter of this work presents background information sufficient for the reader that information is excluded from this "Introduction" chapter. In this way, the reader can gain the best understanding of the material by reading this document straight through, feeling free to skip over repetitive portions of published articles that may be present in the introduction sections of each chapter. The tables, figures, and references of each of the published articles has been changed to be continues throughout this dissertation, with the exception of the articles in the appendix section which are presented in their original forms.

This dissertation discusses studies involved with a once promising drug candidate, D-tagatose. After D-tagatose ran out of patent life before being approved for the market it was returned to the lab and re-worked in multiple preclinical animal studies with combination therapy aimed at overcoming some of the undesirable effects of the drug with the goal of creating a new drug entity. During this process a novel and unexpected result was discovered that led to a patent for this drug combination based off animal data from multiple mammalian models presented here. This combination labeled BSN272 is now working its way through the regulatory framework of obtaining an IND and beginning its first-in-human (FIH) study.

The process of guiding a drug candidate through the approval process in anything but straightforward. If a molecule shows activity in a dish, an animal model, or is discovered to have clinical action through human exposure in a natural setting, it is the job of the development team to position the drug candidate in an environment that gives it the best chance for it to succeed. Succeeding means approval by the Food and Drug Administration (FDA) in the form of a New Drug Application (NDA) which is a process of regulatory action. It is the job of the FDA to deny drug approval as an act of safety to the public, not to do the job of the drug development team in suggesting disease states to target, animal models to pursue, or even a marker of efficacy to evaluate for a treatment that has shown activity, although they will provide some help with this when they can. Ultimately, it is up to the development team to scour the literature, consult experts and specialists at various points, conduct the necessary safety and efficacy studies, comply with drug quality regulations through Good Manufacturing Practices (GMP) tests and documentation (GDP), choose a disease with the best chance of surviving the approval process, taking a drug back into animal studies after clinical trials when appropriate to learn more and tweak, and then take back into the clinic with more information and a better development plan.

This dissertation presents the path of such a process for BSN272, the combination of D-tagatose and trans-polydatin, up to this point from the clinical development of D-tagatose for the treatment of type 2 diabetes, to new pre-clinical studies in hyperlipidemic mammalian models with synergistic actions of trans-polydatin, to choosing the disease state of Prader-Willi Syndrome to best highlight the potential benefits of the drug, and the pre-approval to begin an exploratory, first in human PK study with BSN272 in healthy volunteers.

1.1 D-tagatose

1.1.1 GRAS

D-tagatose is a naturally occurring epimer of fructose at the C-4 carbon found in small amounts in dairy products as an isomerization product of galactose. It was originally studied and patented as a low calorie food sweetener with a reported zero net energy contribution.[1] Various pre-clinical

safety and toxicity studies as well as a handful of small human studies were conducted in order to obtain a Generally Recognized as Safe (GRAS) indication by the FDA for use as a food additive which was granted.[2,3] During this process D-tagatose was tested for safety in a study with type 2 diabetic (T2DM) participants as they would be likely consumers of a low calorie sweetener. While the design of the study was to evaluate the safety in regards to worsening of their blood glucose disorder, it was observed that the patient's blood glucose returned to normal after a 75g oral glucose tolerance test more quickly than those given sucrose instead of D-tagatose.[4,5] This led to a series of small human studies to see if D-tagatose could possibly have any efficacy as an anti-hyperglycemic medication for diabetics.[6,7] These initial studies were positive and Dr. Lodder's lab at the University of Kentucky was asked to assist with putting together a package with which to ask the FDA for an IND to pursue D-tagatose as a treatment for lowering blood sugar in type 2 diabetics. Most of the safety and toxicology studies had already been done to obtain the GRAS status and some efficacy had been shown at doses used for the earlier human studies in T2DM patients. So instead of granting permission to move forward with a Phase I study, the FDA asked for a Phase III study with a large number of T2DM patients powered to achieve efficacy results for the ability of D-tagatose in lowering of HbA1c.

1.1.2 Large-Scale Clinical Trials with D-tagatose for Treatment of Hyperglycemia

An Investigational New Drug (IND) approval by the FDA is required to ship investigational drugs across state lines in order to perform clinical trials. Technically, a human trial can be conducted without an IND if the investigational drug is not shipped across state lines, the trial must simply be approved by the Investigational Review Board (IRB). In the 90's this was common and numerous small scale human trials were conducted with D-tagatose to assess its safe use as a food additive[4,8–14] for which is was given Generally Recognized as Safe (GRAS) status by the FDA[2,15] and many other foreign regulatory bodies. This led to small clinical trials to determine if D-tagatose could be efficacious as an anti-hyperglycemic medication in T2DM patients.[5–7,16,17] In the regulatory world of clinical trials with investigational new drugs today, the IRB's most often require an IND before they will approve any study, regardless of the need (or not) for the drug to cross state lines.

These studies provided positive outcomes and the decision was made to pursue an IND with an indication for treating hyperglycemia. The protocol submitted was for a Phase 1 study to evaluate pharmacokinetics (PK) and safety of D-tagatose, but the FDA responded with an okay to conduct a Phase III large-scale clinical trial powered to determine efficacy. This was due to the unique number of trials and data that D-tagatose had for a first submission of an IND evaluating safety and efficacy. Dr. Lodder was recruited as the PI to plan and conduct this Phase III trial (and subsequent Phase II trial) due to his knowledge of the regulatory arena of pharmaceutical development.

Both of these studies (Phase III and Phase II) were completed just prior to my joining the lab. I had the opportunity to become familiar with D-tagatose, T2DM, and regulation as I co-wrote these papers for academic submission. The Phase III article published in the *Journal of Endocrinology Diabetes & Obesity* is presented in Appendix A (with formatting changes to match this document). In this study 15 g of D-tagatose administered three times a day proved safe and efficacious for the treatment of hyperglycemia in the subjects involved as seen by significant lowering of HbA1c values at all post-baseline time-points in the treatment group. Of some concern was a trend of increased triglycerides in the treatment group towards the end of the study which is addressed by subsequent studies presented here. D-tagatose was well tolerated.

The second was a Phase II trial with three different doses of D-tagatose in T2DM patients in an attempt to further establish the lowest effective dose of D-tagatose that is able to lower HbA1c by $\geq$ 0.5% over a 6-month period.[18] This study is presented in Appendix B. Using each patient's own HbA1c to compare, participants were given 2.5, 5.0, or 7.5 g of D-tagatose three times a day for 6 months with blood levels drawn throughout and measured for various variables that are known to be altered in the T2DM population. 5.0 g of D-tagatose three times a day was the lowest effective dose at lowering HbA1c in this study.

1.2 Physiology of Type 2 Diabetes Mellitus (T2DM)

Type 2 Diabetes Mellitus (T2DM, or type 2 diabetes, or diabetes for this manuscript) is a disorder of blood glucose homeostasis characterized by peripheral insulin resistance and progressive loss of pancreatic beta cell function, that affects more than 400 million people globally.[19] It is also known as non-insulin dependent diabetes mellitus (NIDDM) to distinguish the disease from Type 1 Diabetes Mellitus or insulin dependent diabetes mellitus. In the latter disease, the pancreas of an individual completely loses its ability to produce insulin all at once, sometime usually in childhood, and these patients must administer exogenous insulin to remain alive for the rest of their lives. In T2DM the loss of pancreatic beta cell function and insulin resistance occurs slowly, over decades, and thus these patients can be treated with oral antihyperglycemic agents aimed at improving the various underlying problems, with the goal of avoiding the need for exogenous insulin therapy, which is an eventuality in most cases and a point of no return for these patients.[20] D-tagatose is an example of an oral antihyperglycemic agent and for the remainder of this manuscript any reference to diabetes is regarding the type 2 variety.

To understand the pathophysiology of T2DM we must first discuss glucose homeostasis in a healthy individual. In the fasted state of a healthy individual blood glucose concentrations stay between 80-100 mg/dl. Upon consumption of a meal the glucose concentration rises to 140-180 mg/dl. This increase in glucose concentration signals the pancreatic beta cells to release insulin into the bloodstream. Insulin activities glucose transporters in the periphery to uptake the glucose from the blood which is eventually stored as glycogen to be used later for energy.[21]

In T2DM fasting glucose is maintained above 120 mg/dl and glucose in the fed state increases to well above 180 mg/dl, often reaching 250+ mg/dl. Glucose homeostasis is disrupted because of the inability of the body to maintain it at a normal level with insulin. The 2 hallmarks of this disruption is progressive loss of pancreatic beta cell function in releasing insulin and progressive resistance to insulin by peripheral tissues.[21]

The consequences of long standing hyperglycemia fall into two categories; microvascular and macrovascular complications. Microvascular complications are the major causes of morbidity and include retinopathy, neuropathy, and nephropathy. While these are often the focus of treatment for diabetics, it is the macrovascular complications of the disease that are the major causes of mortality. Heart attack and stroke accounts for almost 80% of all deaths in the T2DM population.[21] The treatment of diabetic dyslipidemia will be a major effort of BSN272 as we will see later in this manuscript.

1.2.1 HbA1c

While blood glucose elevation is the main characteristic of T2DM, it makes for a poor diagnostic. The disease can be diagnosed with 2 fasting blood glucose readings > 120 mg/dl, however, too many things affect blood glucose acutely making the test susceptible to false positives. Instead, practitioners use hemoglobin A1c as a proxy for the blood glucose environment over an extended period of time. Hemoglobin can become irreversibly glycosylated in the presence of glucose. This

process occurs opportunistically and thus is a direct reflection of the glucose environment that the hemoglobin is in. Because the lifespan of a red blood cell is around 3-4 months, the percentage of hemoglobin that is glycosylated can be thought of as representing the glucose environment of the bloodstream over the previous 3 months. HbA1c < 6% is normal. Diabetes is diagnosed at HbA1c > 6.5%.[22]

A major treatment goal for oral anti-hyperglycemic agents is reductions in HbA1c as we will see in many of the studies presented here. These reductions change clinical outcomes as for every decrease in HbA1c by 1% the likelihood of microvascular complications is reduced by 25-35%.[21]

1.3 D-tagatose in Treating Diabetes

A brief introduction to T2DM has been previously presented. What has yet to be discussed is the scope and impact of T2DM specifically in the United States, and the current approaches to treatment. The Center for Disease Control and Prevention determined from records from 2009-2012 that 37% of U.S. adults 20 years of age or older had a conditional known as prediabetes, as did over half of adults over the age of 65. Prediabetes is a condition of hyperglycemia trending towards diabetes but not yet high enough to classify patients as having T2DM. These patients are still at higher risk than healthy individuals for developing heart disease or having a stroke. Treatment at this stage is limited to changes in lifestyle including diet and exercise, with pharmaceutical treatment limited to the comorbidities that may be present such as high blood pressure or hyperlipidemia.[23] If a patient progresses to T2DM usually one or two oral anti-hyperglycemic drugs are added.[24] In 2012, according the American Diabetes Association, $245 billion was spent on patients that had been diagnosed with T2DM, which didn't include an estimated 8 million people that were undiagnosed.[25]

There has been a large introduction of new medications into the market over the last decade for the treatment of T2DM, however, it is estimated that over 60% of patients continue to have HbA1c levels above target goals even with aggressive therapies.[24] A recent review of 140 controlled trials and 26 observational studies concluded that the long-term benefits and harms for currently available treatments remains unclear.[26] It is clear that there remains a need for medications in this disease state both as monotherapy and adjuvant therapy with existing regimens. Moreover, many of the current pharmaceuticals have undesirable side effect profiles, such as hypoglycemia, weight gain, GI distress, or even more serious adverse events such as liver damage or pancreatitis.[27,28] Preferably, such a drug would have a unique mode of action to make it useful as an additive to current therapies and possibly synergistic with them, produce no hypoglycemia, weight gain, or other dose limiting side effect encouraging its use early in the disease process, and aid in resolving any co-morbidities associated with the disease such as atherosclerosis, hyperlipidemia, and cardiovascular disease in general.

As previously mentioned, D-tagatose had undergone a number of preclinical experiments in mice, rats, hamsters, cell culture, and clinical experiments in healthy and diabetic humans. From these experiments and exposures, a mechanism by which D-tagatose produces its anti-hyperglycemic effects has been proposed, although not definitively so. D-tagatose appears to be metabolized through the exact same pathway as D-fructose.[6] After oral administration D-tagatose is absorbed into the bloodstream from the intestines and shuttled to the liver where it is phosphorylated to D-tagatose-1-phopshate by fructokinase. This metabolite (D-tagatose-1-phosphate) has been shown in isolated rat hepatocytes to increase glucokinase activity,[29,30] which phosphorylates glucose to glucose-6-phosphate facilitating removal of glucose from the blood by the liver and increasing glycogen synthesis.[31] An additional shift to glycogen synthesis occurs from an increase of fructose-1-phosphate. While this has not been shown directly for D-tagatose-1-phosphate, co-

administration of D-tagatose and fructose, which happens when D-tagatose is consumed with a meal, causes an increase in fructose-1-phosphate through competitive inhibition of the same metabolizing enzymes for both carbohydrates.[6] Furthermore, cleavage of D-tagatose-1-P by aldolase occurs at about half the rate as it does for the same actions on fructose-1-P. This again causes an increase in fructose-1-P concentrations.[32,33] The increase of glycogen synthesis and inhibition of glycogen utilization is thought to at least in part explain the anti-hyperglycemic effect of the molecule (Figure 1.0).

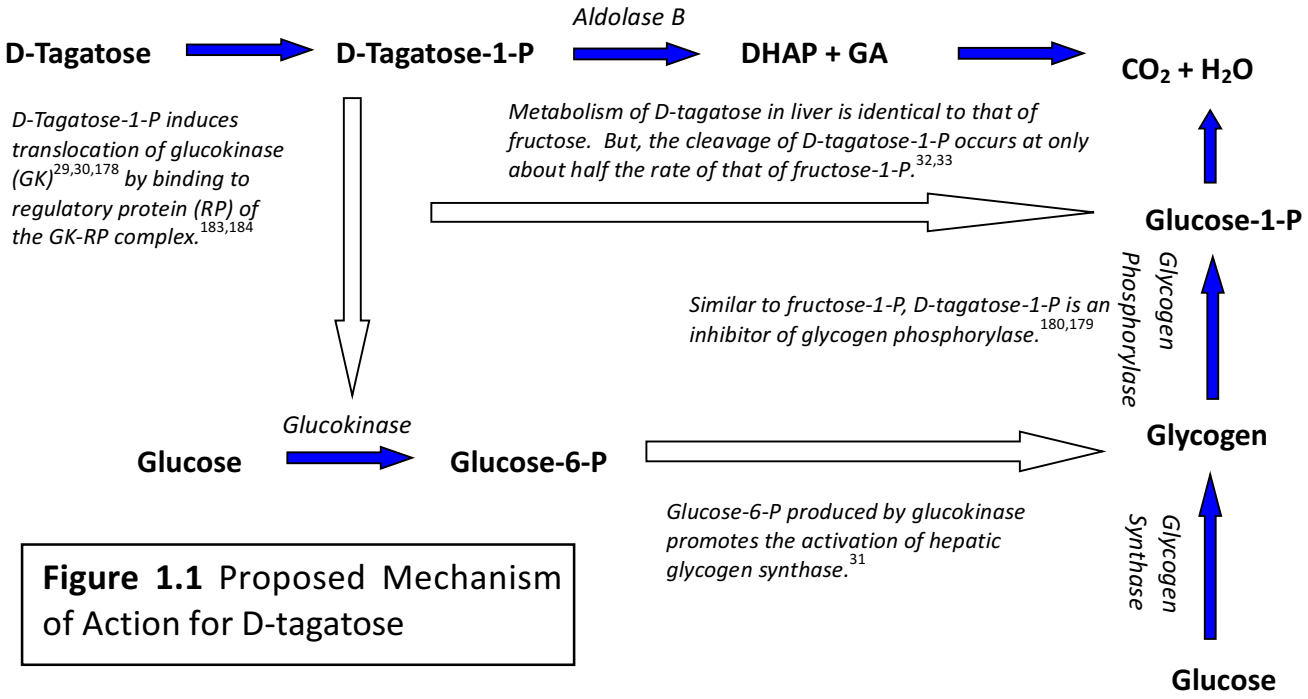


Figure 1.1 Proposed Mechanism of Action for D-tagatose

1.3.1 Effect of D-tagatose on Co-Administration with Other Carbohydrates

It is known that carbohydrates have variable absorption when consumed in isolation and when co-administered with other carbohydrates, as would be present if given D-tagatose with a meal. Notwithstanding the experiments that led to the proposed mechanism of action in Figure 1.0, there was the possibility that the main driver of reductions in blood sugar with administration of D-tagatose was actually due to its inhibition of absorption of other carbohydrates when given with a meal. Conversely, co-administration of glucose with fructose has been shown to dramatically reduce the number of "poor-absorbers" of fructose, meaning that D-tagatose could potentially increase the absorption of fructose when administered together.[34] Therefore, it was pertinent to determine what effect if any D-tagatose administration would have on the absorption of fructose, as the method of administration in practice was likely to be with meals often containing high amounts of fructose.

In Chapter 2 a study is presented in which radiolabeled [^{14}C]-fructose was administered with and without D-tagatose to male SD rats in order to determine the effect of administration of D-tagatose concomitantly with fructose. 2000 mg of D-tagatose per kilogram of rat weight displayed the ability to inhibit fructose absorption as measured by scintillation counts. However, 600 mg/kg of D-tagatose did not have a significant effect on fructose absorption, and this is the dose deemed comparable to the 15 gm dose used in the Phase III study according to FDA recommended BSA

normalization conversion factor.[35] Furthermore, the reduction did not appear to be dose dependent as 6000 mg/kg was not able to further inhibit fructose absorption. This led to a conclusion that the inhibition of [^{14}C]-fructose was due to intolerance of the carbohydrate load as evident by osmotic diarrhea present in the rats.[36] A more thorough discussion of this study and the evidence leading up to it is presented in Chapter 2.

1.4 Approval Problems and Back to the Bench

The Phase II and III trials for D-tagatose resulted in positive outcomes in the T2DM population. However, the landscape for oral anti-hyperglycemic medications is crowded, so the FDA asked for an additional 2 Phase III studies as well as a long-term cardiovascular toxicity study in order to grant and NDA. The original patent on D-tagatose was obtained for its use as a sweetening alternative, so work on its medical use development began late in its patent life and it had only 2 years left by this time. It was, therefore, not feasible to spend the money on all those additional studies without protection from a generic alternative undercutting the market.

This warranted a re-examination of the data from the D-tagatose studies to see if there were any other avenues for the drug that we could pursue. In addition to glucose and HbA1c measurements, there were secondary aims in the Phase II and III studies that were geared towards blood cholesterol and lipid profile effects of D-tagatose. There was some indication of a positive effect towards these aims which would be highly beneficial in the T2DM population that is at an increased risk for developing cardiovascular disease. A concern, however, was a possible increase in triglycerides witnessed in the Phase III trial for patients on D-tagatose compared to placebo (Splenda).[37]

It was hypothesized that the addition of an agent to D-tagatose with the ability to lower or maintain triglyceride levels could produce a product able to treat a myriad of developments of T2DM; hyperglycemia, hyperlipidemia, hypercholesterolemia, and the development of atherosclerosis. Traditionally these are categorized and treated separately even though they have common etiologies and with pathways that overlap. A search of the literature revealed 2 candidates that could fulfill this role; trans-polydatin and dihydromyricetin.

1.4.1 BSN272 in LDLr$^{-/-}$ Mice

Hyperglycemia is the hallmark manifestation of type 2 diabetes mellitus (T2DM). However, macrovascular complications from long standing hyperglycemia, such as heart attacks and stroke, lead to 80% of the deaths reported in this disease state.[21] In fact T2DM has been designated a cardiovascular disease (CVD) equivalent and Haffner *et al.* reported that the 7-year incidence of myocardial infarction in T2DM patients is equal that of non-diabetic individuals that had previously had a heart attack.[38] This was an important consideration for D-tagatose because previous groups have shown that diets high in carbohydrates lead to elevated circulating cholesterol,[39–42] which increases the risks of developing cardiovascular disease. D-tagatose being a carbohydrate similar in structure to fructose, investigations into the possibility of administration of D-tagatose as a therapeutic drug actually contributing to hyperlipidemia and/or atherosclerosis were necessary and were conducted.

Merkel *et al*. had previously demonstrated that LDLr$^{-/-}$ mice fed a diet enriched with sucrose resulted in more elevated LDL cholesterol and resulting atherosclerosis than did a diet enriched with an energy matched equivalent of free fatty acids.[43] Police *et al*. thus conducted a similar study with D-tagatose to see if it had a similar effect. LDLr$^{-/-}$ mice were fed normal diet (control), diet high in sucrose, or diet high in D-tagatose for 16 weeks. Consistent with other studies, the sucrose group led to significant increases in VLDL, LDL, and formation of atherosclerotic plaques

compared to control group. Replacement of sucrose in the diet with D-tagatose did lead to minor elevations in these same parameters (VLDL, LDL, and plaque) but was drastically lower than the sucrose group.

These results were enough to warrant monitoring of the lipid profile of patients during the Phase III and II trials of D-tagatose in T2DM patients as secondary aims. The Phase III study was described previously and the publication is available in Appendix A. LDL and total cholesterol were slightly but significantly reduced in the D-tagatose group compared to placebo from month 6 on in the ITT population, although there was also a slight but significant reduction in HDL cholesterol. Of more concern was a somewhat robust elevation in triglycerides in the D-tagatose group that became significant in month 8 and reached its peak at the completion of the study.[37] These results were concerning given the CVD risks, outlined above, in this population. Additionally, the phenotype of dyslipidemia in the T2DM population favors increased circulation of triglyceride-rich lipoproteins (TRL), as opposed to increased LDL as seen in the general hyperlipidemic population, which have increased atherogenic potential. Recent research has shown that statins fail to correct these features present in diabetic dyslipidemia, and the addition of niacin or fibrates to statin therapy show no benefit.[44]

The first combination study with a lipid lowering therapy involving D-tagatose was with a derivative of trans-resveratrol known as trans-polydatin (or polydatin). The studies were part of a collaboration between labs in the pharmaceutical sciences and physics departments at the University of Kentucky. The initial study involved administering doses of D-tagatose and polydatin to $LDL^{-/-}$ mice fed a high fat, cholesterol, and carbohydrate (Western) diet in a modeling system that develops dyslipidemia and atherosclerosis. The treatment group was sub-sectioned into multiple groups that were given different combinations of doses of D-tagatose and polydatin that were forced to be uncorrelated using principal axis theorem. This allowed the efficacy of each component of the treatment (D-tagatose or polydatin) to be evaluated independently while being in the presence of the other component. In this way, a computer algorithm could be developed that could predict the effects of changing the dose of either component in order to fine tune the treatment.[45] I had the opportunity to contribute to writing this first study using the combination of D-tagatose and trans-polydatin (labeled BSN272) for publication and it is presented in Appendix C.

BSN272 administration to $LDLr^{-/-}$ mice significantly prevented the development of increased VLDL and LDL cholesterol as well as formation of atherosclerosis. There was also a trend towards prevention of increased triglycerides, although it was not significant.

The goal of the collaboration was to create a computational model of a complex system that is dynamic and allows for changes of inputs in real-time with predictable results. In this case the aim was to use the results from a trial in mice measuring changes in lipid levels and atherosclerosis formation when administered D-tagatose, trans-polydatin, and the combination of the two molecules, to create a model of the complex metabolic disease pathway that could simulate the various results that could be expected by differing doses of D-tagatose and trans-polydatin. This was known as a Dynamic Data-Driven Application Simulation (DDDAS).

The modeling system that was developed successfully predicted the results of the study as the molecules were fed to $ApoE^{-/-}$ mice for 8 weeks. BSN272 fed mice showed significantly reduced total cholesterol and VLDL and increased HDL compared to control group. The combination also showed a difference in LDL and triglycerides, however not significantly. This was thought to be due to insufficient group numbers and it was hypothesized that a greater number of subjects would have led to significant results based on the trends seen.[46]

In both studies, however, the lowering of triglycerides appeared to be synergistic as neither component of BSN272 as monotherapy showed any activity towards triglycerides. This set the stage further studies presented in Chapter 4 and summarizes later here in Chapter 1.

1.4.2 BSN723T in ApoE$^{-/-}$ Mice

Dihydromyricetin (DMY) is a flavonoid found in many plants that is used in medicinal teas in Eastern culture for treatment of hang-overs and other ailments. It has recently been investigated in a clinical trial for patients with non-alcoholic fatty liver disease. Patients in that trial developed lowered LDL cholesterol levels.[47] A subsequent study in which an extract from plants that DMY is derived from was administered to rats reduced total cholesterol and triglycerides, as well as increased HDL cholesterol, reported that a drink of the same extract given to humans had similar results.[48] In Chapter 3 a study is presented in which dihydromyricetin and D-tagatose (BSN723T) were added to the food of ApoE$^{-/-}$ mice (genetically modified to produce hyperlipidemia and atherosclerosis) to determine the ability of the combination of the two agents to prevent diet induced increases in triglycerides, total cholesterol, and ultimately the formation of atherosclerotic plaques.

While dihydromyricetin and D-tagatose alone were able to prevent some of the diet induced increases in total cholesterol and atherosclerotic plaques, and prevent triglyceride increases to what would be expected from wild type mice, the combination of the two agents (BSN723T) were no more effective than monotherapy.[49] There appeared to be no synergistic effects between the molecules and this study presented a good comparator study to display the unique potential of BSN272 to warrant further study for that combination.

1.4.3 16 Week ApoE$^{-/-}$ Mice Study with BSN272

The unfortunate finding of increases in triglycerides in the Phase III trial persisted, however, as BSN723T did no better at reducing serum triglycerides than D-tagatose in the ApoE$^{-/-}$ 8-week study. Furthermore, an unpublished study using Syrian golden hamsters showed that polydatin monotherapy greatly increased triglyceride levels. However, BSN272 dramatically reduced triglyceride levels despite a lack of activity towards this measurement for D-tagatose or polydatin alone (unpublished study reviewed in the Introduction section of Chapter 4). The trend toward reduced triglycerides in the BSN272 8-week study[46] and the significant drop in triglycerides witnessed in the unpublished hamster study warranted a follow-up study with this combination in ApoE$^{-/-}$ mice powered to specifically see if a significant reduction in triglycerides could be obtain in a longer study.

Chapter 4 details a study using ApoE$^{-/-}$ mice as a model of diet induced hyperlipidemia. Mice were fed diets containing BSN272 for 16 weeks and measured for serum levels of total cholesterol, triglycerides, and the development of atherosclerosis in the aortic arch. BSN272 was able to significantly prevent diet induced increases in total cholesterol and the development of atherosclerotic plaques. Most importantly, the diet with BSN272 was able to dramatically reduce triglycerides even significantly below control group levels.[45]

1.5 IND Submission

1.5.1 Toxicology

The last of the first-in-human enabling studies required for an IND submission was a rat toxicology study of polydatin, as D-tagatose already had this from the GRAS studies. Appendix D presents this study which found the only adverse test article-related finding to be chronic active pelvic inflammation and transitional cell hyperplasia in one female given 3000 mg/kg/day. Based on

these results, the no observed adverse effect level (NOAEL) was 1200 mg/kg/day for females and 3000 mg/kg/day for males.[50]

1.5.2 Future Work

Chapter 5 offers conclusions and future work. BSN272 was submitted and granted a patent for its use in treating T2DM and the metabolic syndrome. Prader-Willi syndrome was established as a potential indication for future clinical trials as patients with the disease develop T2DM very young and at an extremely high rate. I have written the IND which is in the process of being submitted, some of which can be reviewed in Chapter 5. Appendix E contains the proposed exploratory Phase 1 microdose study with BSN272 in healthy volunteers that is contained in the IND submission.

Chapter 2 – The Effect of Oral D-Tagatose on Fructose Absorption in a Rat Model[36]

2.1 Preface

A thorough background for D-tagatose was presented in Chapters 1 as well as in Phase II and III clinical studies attached in Appendices A and B. D-tagatose showed the ability, through multiple animal models and clinical studies, to lower blood glucose and HbA1c in the presence of chronic hyperglycemia.[6,18,37] The reduced energy contribution of low energy bulk sweeteners is often due to malabsorption,[16] however, D-tagatose has been shown to be absorbed to a greater extent than other lower energy bulk sweeteners, measured by a 68% conversion of ^{14}C radiolabeled D-tagatose oral dose to $^{14}CO_2$, while maintaining a lower net energy content than sucrose.[1] A mechanism of action had been proposed to explain how D-tagatose derives these outcomes which has been pieced together from *in vitro* and *in vivo* studies and is presented in Figure 1.0.

If D-tagatose indeed is absorbed orally to a greater extent than other low calorie bulk sweeteners and contributes zero net energy to the organism then a thermogenic effect must be present, as previously suggested,[1,51] in which more energy is required to metabolize the molecule than is produced. However, an indirect calorimetry study in humans did not support this claim finding no thermic effect and suggesting that a different mechanism must be in place to explain the lack of net energy.[16] Additionally, Saunders *et al.* used germ-free rats to demonstrate that the majority of $^{14}CO_2$ produced from orally dosed radiolabeled D-tagatose was formed by gut fermentation and the actual intestinal absorption in rats was estimated to only be 20%.[52]

While these findings may help explain some of the net zero energy contribution of D-tagatose it still doesn't explain the effects of reducing blood glucose. In addition, as most of the pre-clinical studies replaced sucrose in the diet of the animals with D-tagatose, there had not been a study on absorption or effects on blood glucose of D-tagatose when co-administered with another carbohydrate, as it would be in practice when consumed with a meal. Previous works in multiple labs have explored the phenomenon that the absorption of carbohydrates vary when administered alone or in conjunction with other carbohydrates.[53] Truswell *et al*. determined that fructose exhibits a saturable effect upon oral absorption. When given 50 g orally of pure fructose, 58 of 100 subjects were classified as poor absorbers while only 19 of these previous poor absorbers were unable to properly absorb 25 g of pure fructose. Interestingly, when glucose was given with 50 g of fructose, the number of poor absorbers of fructose was cut in half.[34] This study was confirmed years later in a rat model in which researchers at the University of California claimed the mechanism of the effect is due to the disaccharidase-related transport system.[54] Prieto *et al*. then demonstrated that the alterations in absorption between glucose alone, fructose alone, and the combination of glucose and fructose, did in fact manifest into changes in glycemia and insulin responses.[55]

In light of these findings and the increasing prevalence of fructose in modern foods, it became important to distinguish the consequence that D-tagatose would have on fructose absorption if it were to be given therapeutically as an anti-hyperglycemic medication. I wrote this paper which was published in the *Journal of Developing Drugs*. The article is presented below with the table and figure numbers changed to be consistent with this dissertation.

2.2 Introduction[36]

A naturally occurring epimer of fructose, D-tagatose is present in small amounts in many dairy products, such as milk, numerous cheeses, and certain kinds of yogurts.[1,56] Interest in D-tagatose as a sweetening alternative to conventional sugar manifested from the finding that it provided no available energy when fed to rats,[51] yet it maintains a sweetness equivalent to 92% that of

sucrose.[1] D-tagatose was subsequently examined and granted US patent approval in 1988, as a low calorie full bulk sweetening agent.[57]

The majority of research in the following years revolved around elucidating the toxicity profile of the substance, with the aim of pursuing FDA approval as a food additive. Multiple *in vitro* and *in vivo* studies were completed with the results establishing that D-tagatose was not genotoxic,[58] no evidence of treatment-related effects were seen at low doses, and at high doses, the transient toxicity of soft stools consistent with osmotic diarrhea (from incomplete absorption of the carbohydrate) occurred.[59,60] Human studies then commenced with results closely mimicking that of the animal models.[8,10] A decade of human, animal and other toxicity and safety data led to D-tagatose gaining "Generally Recognized As Safe" (GRAS) approval by the FDA,[2] allowing its use in food and beverages in limited amounts. No incidence of toxicity has been reported to date from usage in food and beverages. Following this, the European Union (EU) allowed D-tagatose as a 'novel food ingredient', with no restrictions on the amount in which it can be used.[6]

Interest in the molecule as a pharmaceutical agent began as a result of discovering it to have antidiabetic properties in animal models.[61] Previous GRAS approval allowed prompt entry of D-tagatose into human studies as an antihyperglycemic agent. Early results indicated that there was no change in glucose or insulin levels, following oral administration of D-tagatose in the fasted state.[7] Even more interestingly, D-tagatose showed the ability to blunt the rise in blood glucose, when given prior to oral glucose intake.[4] These studies conducted at the University Of Maryland School Of Medicine and subsequently confirmed at the Research Department of Nutrition, Copenhagen[9] led to the consensus that D-tagatose held the potential of becoming an adjunct therapy for patients with Type II *Diabetes mellitus*.

In 2010, a phase II dose ranging study over 6 months and a phase III efficacy study over 12 months were completed using D-tagatose to reduce HbA1c in 161 and 494 patients, respectively, in the US and in India (clinicaltrials.gov identifier NCT00961662 and NCT00955747). The results showed statistically significant reductions in HbA1c compared to placebo, with the only adverse effects being of the GI variety, consistent with prior studies. The exciting results of these studies offered promise for D-tagatose as a diabetic medication and warranted further examination into how D-tagatose achieves its effects, and how it interacts with other components of the human diet, especially other carbohydrates.[53]

Previous work in multiple labs has explored the variable absorption of different carbohydrates when administered alone, or in conjunction with other carbohydrates. Truswell *et al*.[34] determined that fructose exhibits a saturable effect upon oral absorption. When given 50 g orally of pure fructose, 58 of 100 subjects were classified as poor absorbers, while only 19 of these previous poor absorbers were unable to properly absorb 25 g of pure fructose. Interestingly, when glucose was given with 50 g of fructose, the number of poor absorbers of fructose was cut in half. This study was confirmed years later in a rat model, in which researchers at the University of California claimed the mechanism of the effect is due to the disaccharidase-related transport system.[54] Prieto *et al*.[55] then demonstrated that the alterations in absorption between glucose alone, fructose alone, and the combination of glucose and fructose, in fact manifested as changes in glycemia and insulin responses.

In light of these findings and the increasing prevalence of fructose in modern foods, it became important to distinguish the consequences of D-tagatose on fructose absorption, if it were to be given therapeutically as an antihyperglycemic medication. The present study aimed to provide new information D-tagatose consumption with fructose through administration of ^{14}C labeled fructose and D-tagatose concomitantly, and comparing the radioactivity present in plasma

samples collected at various time points to that of fructose monotherapy in a rat model. We first completed a pilot study using [^{14}C]-fructose with glucose, to establish the viability of our procedure to measure changes in carbohydrate absorption, and subsequent plasma levels, and to derive the amount of radioactivity that would be needed in the fructose batch to obtain interpretable scintillation count data. From the results of this initial study, we aimed to establish the effect that a graduated change in concentration of D-tagatose would have on absorption of 2 g/kg of fructose, with a secondary aim of determining how the resulting absorption would manifest in terms of blood glucose.

2.3 Materials and Methods[36]

Animals

Male Sprague-Dawley FVC (FVC = femoral venous catheter) rats from Harlan weighing 275 to 300 g were used for these studies. The Sprague-Dawley FVC rats had femoral vein cannulas (implanted by the animal vendor) tunneled under the skin that exit superior to the scapula. Catheters were checked for patency and maintained with heparin + glycerol (1000 IU/ml). Patency checks were conducted on the day of receipt and at regular intervals until use. Locking solutions were composed of an equal mixture of USP grade glycerol and heparin (1000 IU/ml). Rats were housed in solid bottom cages with bedding in ventilated stainless-steel racks and were individually caged to prevent cage mates from gnawing on the cannulae. The rats were placed in a normal light cycle, 12 hours light and 12 hours dark, and were housed for 3 to 7 days in a vivarium prior to testing. Animals were conditioned to hooded restrainers for approximately 15 min on days (-5 to -3) and (-3 to -1) prior to the start of the study.

All animal care and procedures of the study were performed in compliance with the U.S. Department of Agriculture's (USDA) Animal Welfare Act (9 CFR Parts 1,2, and 3); the Guide for the Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources, National Academy Press, Washington, D.C., 1996; and the National Institutes of Health, Office of Laboratory Animal Welfare.

Plasma [^{14}C]-Fructose Measurement Following Oral Administration of a Bolus [^{14}C]-Fructose/Glucose Preparation

Two Fructose/Glucose dosing solutions containing either 250 uCi/g or 83.5 uCi/g [^{14}C]-Fructose were prepared to deliver 2 g/kg of each sugar via oral gavage. Radiolabeled fructose came from American Radiolabeled Chemicals, St. Louis, MO. Two additional dosing solutions containing either 250 uCi/g or 83.5 uCi/g [^{14}C]-Fructose were prepared to deliver 2 g/kg Fructose alone via oral gavage. Four groups of rats (8 rats per group) were dosed as follows: Group 1 – 2000 mg/kg [^{14}C]-Fructose (250 uCi/g); Group 2 - 2000 mg/kg [^{14}C]-Fructose (250 uCi/g) + 2000 mg/kg Glucose; Group 3 – 2000 mg/kg [^{14}C]-Fructose (83.5 uCi/g); Group 4 - 2000 mg/kg [^{14}C]-Fructose (83.5 uCi/g) + 2000 mg/kg Glucose. At 0, 1, 3, 5, 10, 15, 30, 45, and 60-minute time points, whole blood was obtained from the venous catheter, measured for total glucose, centrifuged, and plasma retained for scintillation counting of ^{14}C.

Effect of D-tagatose on Plasma [^{14}C]-Fructose Absorption Following Oral Administration of a Bolus [^{14}C]-Fructose/D-tagatose Preparation

A Fructose dosing solution containing 250 uCi/g [^{14}C]-Fructose was prepared to deliver 2 g/kg of fructose alone or with either vehicle, 0.6 g/kg, 2 g/kg, 6 g/kg, or 15 g/kg D-tagatose via oral gavage. Five groups of rats (8 rats per group) were dosed as follows: Group 1 - 2000 mg/kg [^{14}C]-Fructose (250 uCi/g); Group 2 - 2000 mg/kg [^{14}C]-Fructose (250 uCi/g) + D-tagatose 600 mg/kg; Group 3 -

2000 mg/kg [^{14}C]-Fructose (250 uCi/g) + D-tagatose 2000 mg/kg; Group 4 - 2000 mg/kg [^{14}C]-Fructose (250 uCi/g) + D-tagatose 6000 mg/kg; Group 5 - 2000 mg/kg [^{14}C]-Fructose (250 uCi/g) + D-tagatose 12000 mg/kg. At 0, 1, 3, 5, 10, 15, 30, 45, 60-minute time points whole blood was obtained from the venous catheter, measured for total glucose, centrifuged, and plasma retained for scintillation counting of ^{14}C.

Sample Analysis

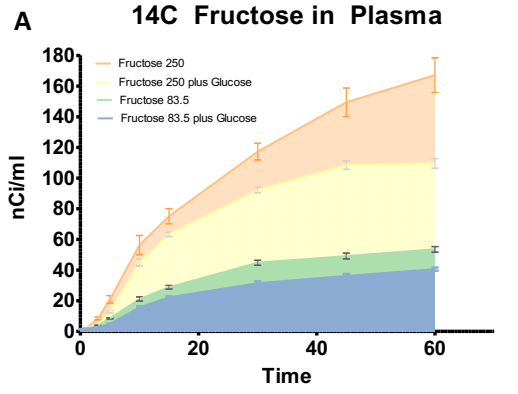
Blood samples were analyzed for total glucose using a MediSense™ Precision PCx® Glucometer (Model M6012-0014) with MediSense™ Precision PCx® Glucose Test Strips (Model 99565-01). Scintillation counting for ^{14}C was conducted on 10-30 uL of plasma in 10 mL of Fisher ScintiSafe Gel (SX24-5) scintillation fluid, using a Beckman scintillation counter (Model LS6500).

2.4 Results[36]

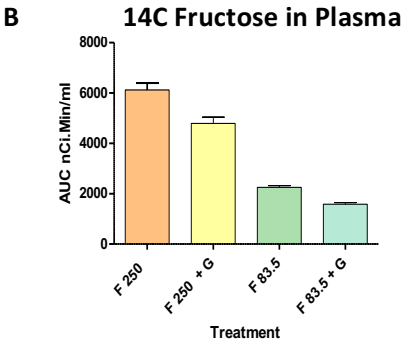
The pilot study aimed to set the level of radioactivity to be used later in the fructose/D-tagatose portion by use of a well-known pairing (glucose:fructose) and to verify that changes in systemic carbohydrate levels could be measured accurately using this procedure. Figure 2.1A shows the accumulation of radioactivity in the plasma at various time points. The AUC for each group is superimposed over each other and the amount of activity in nCi/ml over the 60-minute study is displayed. Addition of glucose to fructose in equal amounts (2 g/kg weight of rat) resulted in a decrease in plasma fructose of 26% in the 250 uCi/g group and 27% in the 83.5 uCi/g group. Figure 2.1B compares the effect glucose had on fructose absorption. Administration of glucose with fructose resulted in a mean difference of 1634 and 600 nCi·hr/ml in the 250 and 83.5 uCi/g of fructose groups, respectively, and reached significance as calculated by the Bonferroni's Multiple Comparison Test.

Figure 2.1[36]

^{14}C Scintillation Counts in Plasma, Pilot Study Results



	Fructose 250	Fruc 250 + Glucose	Fructose 83.5	Fruc 83.5 + Glucose
Total Area	6382	4748	2246	1646



Bonferroni's Multiple Comparison Test	Mean Diff.	Significant? P < 0.05?	Summary
F 250 vs F 250 + G	1325	Yes	***
F 83.5 vs F 83.5 + G	675.8	Yes	*

A: Radioactivity measured in nCi/mL is plotted over the 1-hour study for each of the 4 arms. The AUC for each group is also included in the table below the figure.[36]

B: Display of the relative differences between AUC of the different treatment arm measured in nCi*hr/ml. The table below the figure gives the numerical values for these differences.[36]

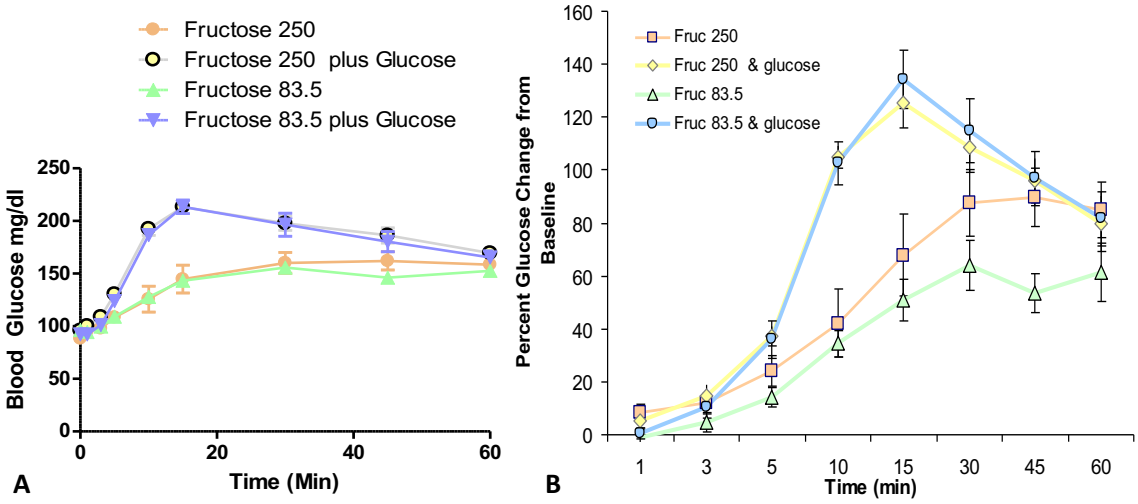
Figure 2.2A compares the different treatment arms with respect to blood glucose throughout the study. Administration of fructose via oral gavage resulted in an increase in glucose levels in all arms of the study. Figure 2.2B shows the change in glucose concentration from baseline (taken at time 0 before administration of treatment). Oral fructose resulted in a change in blood glucose

that peaked at a concentration of 80 mg/dl in the fructose alone groups. When glucose was given with fructose the amount of glucose in the blood peaked near 140 mg/dl. Additionally, the time to peak glucose concentration occurred at the 30 or 45-minute time point in the fructose alone arms and at the 15-minute time point in the fructose/glucose co-administration groups.

The results of the pilot study established that 250 uCi of radioactivity per gram of fructose would provide adequate scintillation counts to measure a change in fructose absorbance with co-administration of another carbohydrate. The following figures document the simultaneous intake of [^{14}C]-Fructose with 250 uCi/g of radioactivity administered at 2g/kg of body weight with either vehicle, 0.6 g/kg, 2 g/kg, and 6 g/kg of D-tagatose via oral gavage. Figure 2.3 shows the effect that tagatose had on the AUC of radioactivity from [^{14}C]-Fructose in the plasma over the 60 minutes of the study. Figure 2.3A represents this by overlapping the graphs of AUC from the different treatment arms. Figure 2.3B shows that 600, 2000, and 6000 mg of D-tagatose per kg of body weight decreased the AUC of fructose absorption by 71 (1% decrease), 1801 (26% decrease), and 2072 (30% decrease) nCi·hr/ml, respectively. The 2000 and 6000 mg/kg doses reached significance whereas the 600 mg/kg group did not (the 12000 mg/kg group was stopped early in the study due to intolerance and 1 fatality).

Figure 2.2[36]

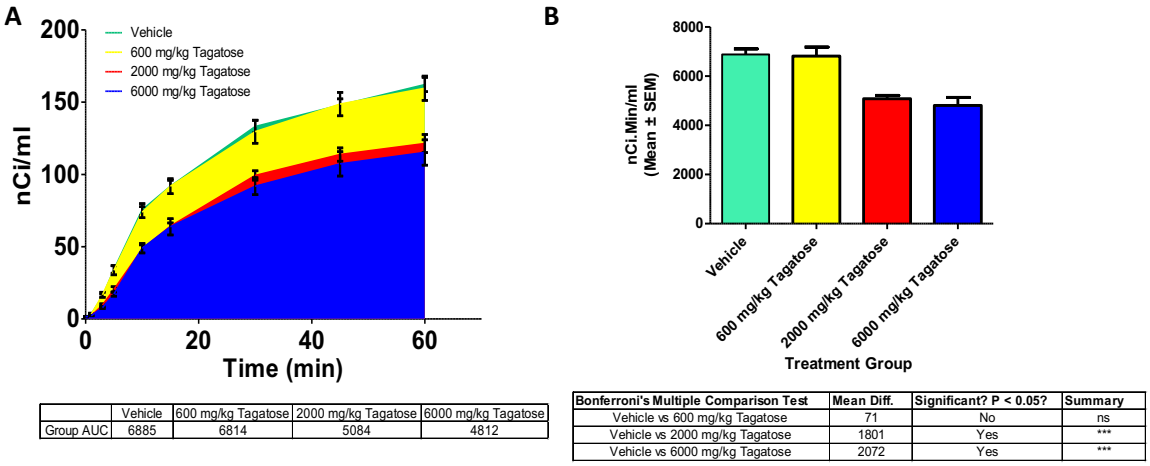
Blood Glucose in Plasma, Pilot Study Results



A: Displays the blood glucose concentrations given in mg/dl at each time point during treatment.[36]
B: Displays the blood glucose readings as a percentage change from baseline.[36]

Figure 2.3[36]

^{14}C Scintillation Counts in Plasma, D-tagatose Study Results



	Vehicle	600 mg/kg Tagatose	2000 mg/kg Tagatose	6000 mg/kg Tagatose
Group AUC	6885	6814	5084	4812

Bonferroni's Multiple Comparison Test	Mean Diff.	Significant? P < 0.05?	Summary
Vehicle vs 600 mg/kg Tagatose	71	No	ns
Vehicle vs 2000 mg/kg Tagatose	1801	Yes	***
Vehicle vs 6000 mg/kg Tagatose	2072	Yes	***

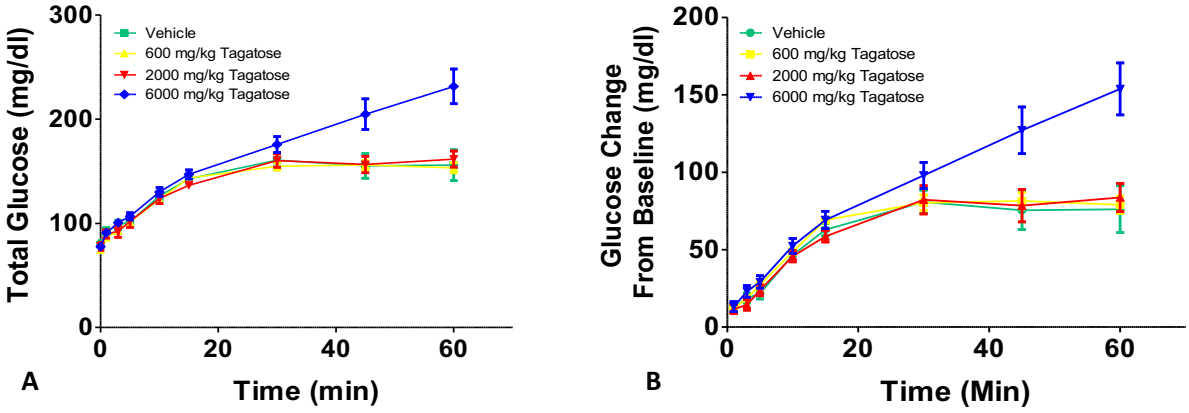
A: Radioactivity measured in nCi/mL is plotted over the 1-hour study for each of the 4 arms. The AUC for each group is also included in the table below the figure.[36]

B: Display of the relative differences between AUC of the different treatment arm measured in nCi*hr/ml. The table below the figure gives the numerical values for these differences.[36]

Figure 2.4 illustrates the effect that blood glucose had on administration of 2 mg of fructose per kg of body weight along with either vehicle, 600, 2000, or 6000 mg/kg of D-tagatose. The 600 and 2000 mg/kg of D-tagatose groups produced blood glucose levels that were no different than with administration of fructose alone (vehicle). At 6000 mg/kg an elevation in blood glucose was seen at the 30-minute time point that continued to rise, reaching 232 mg/dl at the last sampling (minute 60). This represented nearly a 75 mg/dl difference from the vehicle, 600 and 2000 mg/kg groups.

Figure 2.4[36]

Blood Glucose in Plasma, D-tagatose Study Results



A: Displays the blood glucose concentrations given in mg/dl at each time point during treatment.[36]

B: Displays the blood glucose readings as a percentage change from baseline.[36]

2.5 Discussion[36]

The study at hand consisted of two phases. The aim of the pilot portion was twofold: (1) to determine the level of radioactivity in fructose that needed to be present in order to produce

readily measurable scintillation counts in plasma samples and (2) to validate that the procedure that was used could determine changes in the systemic levels of carbohydrates with significance. The second portion of the protocol held the answers to the questions that motivated the study. Here the exercise validated in the pilot study was used to elucidate the effect that D-tagatose would have on absorption of fructose co-administered with it in an in vivo rat model. The effect that the absorbed fructose had on glycemia, or more importantly, the ability of D-tagatose to blunt the glycemic effect of fructose was established.

The pilot study determined that a radioactivity level of 250 uCi/g of fructose was sufficient to accurately measure the amount of fructose in the plasma after oral administration via oral gavage. The amount of fructose present in plasma when administered with equal amounts of glucose was reduced compared to fructose monotherapy. Additionally, fructose administration resulted in hyperglycemia in all arms of the study. Both these findings appear to be controversial in light of other literature,[34,54,55] yet there are multiple reasons this conclusion may be unwarranted. Firstly, the pilot study wasn't designed for external validity of previous studies, only to lay the groundwork for the second part of the fructose/D-tagatose study to follow. For instance, our study lacked a sham group (receiving the catheter but no active substance). Because of this, it is impossible to determine the effect that the procedure itself had on absorption. Boudry et al established in a study conducted in 2007, that stress induced to rats can cause an upregulation of GLUT2 at the apical membrane of the brush border of the intestines and cause a shift from absorption of glucose through SGLT1 to absorption through GLUT2.[62] GLUT2 is a known transporter of glucose, fructose, and galactose in high amounts (low selectivity, high capacity).[63] Even though the animals were preconditioned to the procedure, it's possible that the stress of the procedure itself, or the placement of the catheter, caused an upregulation in GLUT2 at the luminal membrane, which has been documented to occur in the span of minutes.[64] Stress could cause increased absorption of fructose during monotherapy and a reduction in fructose absorption in the presence of glucose, as it competes for transit via the same GLUT2 transporter that is facilitating a major portion of the fructose absorption. Additionally, the lack of a glucose alone group inhibits us from comparing the ability that fructose itself had to decrease glycemia as shown in some of the studies mentioned previously. Again, the pilot study was not designed to be compared to these studies, simply to provide a reference for the second study of the effect that D-tagatose would have on glycemia, when administered with fructose (compared to fructose alone).

In the second portion of the study, we showed that D-tagatose was able to decrease the amount of fructose absorbed when co-administered via oral gavage. This reduction was most prevalent at the 2000 mg/kg dose. This reduction did not seem to be strongly dose dependent. The 6000 mg/kg dose of D-tagatose was only slightly better at inhibiting fructose absorption. We believe this is simply a situation of saturation of carbohydrates. There was no run-in period to acclimate the rats to D-tagatose or fructose. In fact, the 12000 mg/kg dosing group had to be stopped due to fatality. At an acute dose of 6000 mg/kg group and up, and even with 2 g/kg of fructose, the rats seem to be in a state of malabsorption, characterized by osmotic diarrhea and symptoms of overt stress. D-tagatose is an epimer of fructose, and the rats responded to the treatment as if they had been given a single dose of fructose that grossly exceeded their ability to absorb it. Previous studies have shown fructose malabsorption to be around 2 g/kg, so with the addition of D-tagatose, all of our arms were above this range. We also suspect that the stress of malabsorption and correlating symptoms explains the rise in blood glucose in the 6000 mg/kg dosing group.

The mechanism by which D-tagatose is able to reduce HbA1c in diabetic patients is not fully established. The phase 2 and 3 clinical trials showed that with a 2-week run-in period malabsorption could be controlled even with high doses of D-tagatose three times daily, while maintaining HbA1c reduction. In the 1-year phase 3 trial, HbA1c continued to drop month by month throughout the trial with no signs of tachyphylaxis. The present study provides another small piece of the puzzle, and a future study will determine the ultimate fate of dietary fructose in D-tagatose treated patients.

When AUC is used to measure exposure of a drug, integration of peak area is usually performed until drug is completely eliminated from the body. As we were measuring a radioisotope of carbon, it was impossible to do this in our study, because we could not establish when the carbon would have been metabolized from fructose into another molecule. Therefore, the absorption of fructose may have simply been delayed and would have shown up, had we performed a different study over a longer period. A full metabolic study of the fructose in a given dose in the presence of D-tagatose will answer many such questions. Elucidation of the mechanisms of action of D-tagatose may help to optimize an interesting candidate for anti-diabetic therapy, or isolate a population of diabetics in which this drug could be maximally effective.

Acknowledgments

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Chapter 3 - BSN723T Prevents Atherosclerosis and Weight Gain in ApoE Knockout Mice Fed a Western Diet[49]

3.1 Preface

Treating hyperglycemia and hyperlipidemia in T2DM patients runs along the same lines of treating the greater disorder of dysfunctions in energy homeostasis, and it is clear that statins and other currently available anti-lipid medications are unable to properly treat this patient population. The potential became apparent that adding a molecule to D-tagatose aimed at lowering triglyceride levels could be advantageous and possibly even synergistic with the activities of D-tagatose. In the current study the flavonoid dihydromyricetin (DMY) was added to D-tagatose (designated BSN723T) and administered to a hyperlipidemic mouse model.

Dihydromyricetin (designated BSN723 or DMY), also known as ampelopsin, is a flavonoid isolated from a number of plants, such as *Ampelopsis grossedentata*, and used in traditional eastern medicine. DMY has shown positive results for numerous health benefits including inflammation,[65] anti-oxidant,[66] anti-hypertensive,[67] and atherosclerosis.[68] It also appears to be beneficial as a treatment for alcohol intoxication[69] and early studies suggest it may have potential as a treatment for Alzheimer's disease.[70]

What made DMY stand out for combining with D-tagatose was a recent clinical study from Chen *et al*. which looked at the effects of dihydromyricetin on nonalcoholic fatty liver disease (NAFLD). The pathogenesis of NAFLD includes insulin resistance, oxidative stress, mitochondrial dysfunction, and liver inflammation. The study focused on inflammatory mediators and biomarkers of NAFLD but also looked at glucose and lipid metabolism. While DMY was not able to effect fatty infiltrations of the liver, it did improve levels for several liver enzymes, and led to decreased LDL cholesterol levels.[47] A second study reported that a drink made from *Ampelopsis grossedentata* extract given to humans lowered triglycerides and total cholesterol levels.[68]

The decision was made to go ahead with this D-tagatose and dihydromyricetin study on the heels of the successful D-tagatose and trans-polydatin study (Appendix C) in order to have a comparator study of D-tagatose with a lipid lowering therapy. The results of the BSN272 studies appeared to show that D-tagatose and trans-polydatin were synergistic in their effects. As DMY had shown efficacy in lowering of triglycerides and cholesterol as monotherapy it had the potential to offer synergistic effects as well. If the results were that no synergistic effects existed, then that would give more weight to the idea that BSN272 was unique and worth pursuing as a combination therapy. $ApoE^{-/-}$ mice were used in this study instead of $LDLr^{-/-}$ because they develop atherosclerosis more robustly when fed a Western diet. I led the investigation and writing of this study and it was published to *Webmed Central*. The article is presented here with the figure and table numbers changed to be consistent with this dissertation.

3.2 Introduction[49]

Evidence strongly supports a link between obesity and a spectrum of diseases including type 2 diabetes, hypertension, hyperlipidemia, and cardiovascular disease. Hyperlipidemia is commonly seen 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 populations.[71,72] Such secondary 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. Reduction of elevated LDL and raising of HDL have been major drug treatment goals,

and drugs have been developed that alter blood lipids and produce significant reduction in cardiovascular events in patients with cardiovascular disease and diabetes.[73,74]

Elevated serum cholesterol levels have been noted in rodents,[39] dogs,[40] nonhuman primates,[41] and humans[42] consuming a high-carbohydrate diet, particularly one including fructose and sucrose. Studies have provided evidence that fructose causes hypertriacylglycerolemia postprandially both directly through decreased triglyceride clearance, and indirectly by increasing liver re-esterification of fatty acids.[75] Low-density lipoprotein receptor deficient ($LDLr^{-/-}$) 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.[43]

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 human studies and shows promise as a treatment for type 2 diabetes and obesity.[4,17,18,37] After more than 10 years of animal and human studies, 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 serious adverse events reported.[6]

The mechanism by which D-tagatose produces its antihyperglycemic effect in response to a meal is not clear. Based on studies with fructose and D-tagatose, it was proposed that D-tagatose is metabolized following a pathway that is essentially the same as that of fructose.[6] 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[29,30] leading to increased phosphorylation of glucose to glucose-6-phosphate and further activating glycogen synthase.[31] It has been suggested that D-tagatose-1-phosphate can inhibit glycogen phosphorylase in the same manner that fructose-1-phosphate does,[6] 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, at least partly explaining the antihyperglycemic effect of the sugar. In addition to the effect on glycogen regulation, D-tagatose inhibits sucrase,[76] leading to the suppression of sucrose digestion in the small intestine and inhibits the activity of maltase, at least in vitro, which 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.[4–6,13,18,37,58–60,77,78]

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, $LDL^{-/-}$ mice fed a diet in which high sucrose content was replaced with an equivalent amount of D-tagatose exhibited reduced cholesterol, triglycerides, and atherosclerosis compared to mice on the diet containing sucrose.[78] 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.[5] This is interesting in light of evidence suggesting that increasing HDL levels decrease the risk of incurring a coronary event. 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.[4,5]

Dihydromyricetin (BSN723 or DMY), also known as ampelopsin, is a flavonoid that has been isolated from a number of plants, including Ampelopsis grossedentata, Cedrus deodara, Hovenia dulcis, and Erythrophleum africanum, that have been used in traditional medicine. Many claims have been made regarding dihydromyricetin's numerous health benefits including antioxidant properties,[66] anti-cancer,[79–81] anti-hypertensive,[67] anti-inflammatory,[65] and anti-atherosclerotic effects.[68] Dihydromyricetin is also indicated as a treatment for alcohol intoxication[69] and a preliminary study suggests it as a possible treatment for Alzheimer's disease.[70]

A number of published studies provide evidence that dihydromyricetin can protect cells against oxidative injury (for example, see Zhang *et al*. 2003,[66] Ye *et al*. 2008,[82] Lin *et al.* 2014,[83] Zou *et al*. 2014).[84] Recently, Jiang *et al.* (2014)[85] examined the effects of dihydromyricetin on oxidative stress and glucose transport activity in a methylglyoxal (MG)-induced PC12 cell line to explore the possibility of using dihydromyricetin for the treatment of MG-induced diabetes-associated cognitive decline. They found that DMY protected PC12 cells against MG-induced apoptosis and glycometabolic disorders, at least in part by restraining the hyperactivation of p-AMPK activity and normalizing the translocation of GLUT4 from the intracellular compartment, resulting in a balance in glucose uptake.

Much attention has been focused on the use of dihydromyricetin in the treatment of a variety of cancers and there have been many studies, both in vitro and in vivo, demonstrating inhibitory activity of dihydromyricetin against cell lines of breast cancer,[86] liver cancer,[80,81,83] melanoma,[87] osteosarcoma,[88] and lung cancer.[89,90] Dihydromyricetin has also shown anticancer activity against bladder cancer,[91] lung cancer,[92] and prostate cancer[79] xenografts, and showed synergistic effects with adriamycin for treating leukemia xenografts.[93]

Recently, Chen *et al.* (2015)[47] looked at the effects of dihydromyricetin on nonalcoholic fatty liver disease (NAFLD) in a clinical study. The pathogenesis of NAFLD includes insulin resistance, oxidative stress, mitochondrial dysfunction, and inflammation in the liver.

The study looked at inflammatory mediators and biomarkers of NAFLD as well as glucose and lipid metabolism. They found that while dihydromyricetin did not alter the severity of fatty infiltration in the liver, it did produce significant improvements in several liver enzymes and reduced serum levels of several markers including tumor necrosis factor-alpha, cytokeratin-18, and fibroblast growth factor 21. They also found that the HOM-IR level was decreased in dihydromyricetin treated patients, but insulin and C-peptide levels were not affected. Levels of low-density lipoprotein-cholesterol (LDL-C) and apolipoprotein B (Apo B) were also significantly decreased by dihydromyricetin, but the total cholesterol, triglyceride, high-density lipoprotein-cholesterol (HDL-C), and Apo-A-I concentrations did not significantly differ between treated and control groups. Other evidence that dihydromyricetin can affect glucose metabolism include studies that found that DMY activated insulin signaling and increased glucose uptake in skeletal muscle in vitro and in vivo.[94,95]

Recent studies have begun to offer clues about dihydromyricetin's mechanism of action. It is now known that a number of cell signaling pathways are affected by dihydromyricetin. Zou, *et al*. (2014)[84] found that dihydromyricetin fed to rats for 7 days increased the expression of peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) in skeletal muscle. PGC-1α is known to regulate irisin, an exercise-induced myokine that can stimulate the browning of white adipose tissue. In a follow-up study (Zhou, *et al*., 2015)[96] the effect of dihydromyricetin on irisin secretion through the PGC-1α pathway was investigated in vivo (in rats and humans) and in vitro (L6 myotubes). The results were an increase in irisin secretion with the administration of dihydromyricetin.

Dihydromyricetin has also been found to increase the levels of phosphorylated AMP activated protein kinase (AMPK) and Ulk1, and decrease phosphorylated mTOR levels.[94] The same group also found that dihydromyricetin increased levels of peroxisome proliferator-activated receptor coactivator-1α (PGC-1α), and Sirt3 in skeletal muscle in vitro and in vivo.[95] Jiang, *et al*. (2014) found that dihydromyricetin ameliorates the oxidative stress response induced by methylglyoxal via the AMPK/GLUT4 signaling pathway.[85]

A previous study combining D-tagatose with another naturally occurring antioxidant, polydatin, found the combination to be effective in lowering total cholesterol and preventing the formation of atherosclerosis in ApoE$^{-/-}$ mice.[46] There has been relatively little published research regarding DMY combination drugs or dihyromyricetin's effect on cardiovascular health. Chen *et al*. (2015)[47] recently reported that dihydromyricetin lowered LDL cholesterol in a human clinical trial. A second study reported that administering an extract from A. grossedentata to rats reduced serum total cholesterol and triglycerides and increased high-density lipoprotein, and that humans given a drink made from A. grossedentata showed a reduction in serum triglycerides, total cholesterol, and plasma lipid.[68]

In the present study, we aim to examine the effect of BSN723T, a combination of D-tagatose and dihydromyricetin, on blood lipids and atherosclerosis in ApoE$^{-/-}$ knockout mice. The ApoE lipoprotein resides on very low, intermediate, and high density lipoproteins (VLDL, IDL, and HDL, respectively) and mediates the removal of lipoproteins 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 elevated cholesterol and triglycerides in these mice. Severe hypercholesterolemia and the rapid development of atherosclerosis results when these mice are fed a Western type diet consisting of high fat, high cholesterol and high sucrose.[97–99] 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.[98] We tested the hypothesis that BSN723T lowers serum triglycerides and cholesterol, and prevents the formation of atherosclerosis in ApoE$^{-/-}$ mice fed a Western diet. This is the first known study examining the combined effects of D-tagatose and BSN723 on blood lipids and the development of atherosclerosis.

3.3 Methods[49]

Materials

D-tagatose was obtained from Inalco, S.p.A. (Milano, Italy). Dihydromyricetin [(2R, 3R)-3,5,7-trihydroxy-2-(3,4,5--trihydroxyphenyl)- 2,3-dihydro--chromen-4-one] was obtained from Qingdao Ai Weisheng Chemical Co. Ltd. (Shandong, China).

Mice and Diets

The animal-use protocol was approved by the University of Kentucky Institutional Animal Care and Use Committee. Male ApoE$^{-/-}$ mice (C57BL/6 background) 14 to 18 weeks of age were obtained from Taconic Biosciences. The mice were given water and kept on a 12-hour light/dark cycle. Mice were randomized into 5 groups (n = 10 per group) to ad libitum produce groups with the equivalent mean body weights.

Group 1 (Standard diet) is a negative control group for atherosclerosis and was fed Standard chow (TD.2018, see Table 3.1 for diet compositions) for the duration of the study.

Group 2 (Western diet) is a positive control group for atherosclerosis and was fed Standard chow for the two-week D-tagatose run-in period (see below) and then switched to a high fat, high cholesterol, high sucrose, Western diet (TD.88137) for the remainder of the study.

Group 3 (D-tagatose diet) was on Standard chow during the two-week run-in period during which the mice of this group were given gradually increasing concentrations of D-tagatose in the water every day until the total daily mass of D-tagatose drank by the mice matched the mass consumed in the study chow, assuming average daily consumption of water and chow (see Table 3.2). These mice were then switched to a Western diet chow (TD.140143), in which the sucrose was replaced by D-tagatose, for the remainder of the study.

Group 4 (BSN723 diet) was on Standard chow during the two-week run-in period and then switched to a Western diet with dihydromyricetin (BSN723) added (TD.140144) and kept on this diet for the remainder of the study.

Group 5 (BSN723T diet) was on Standard chow during the two-week run-in period during which the mice of this group were given gradually increasing concentrations of D-tagatose in the water every day until the total daily mass of D-tagatose drank by the mice matched the mass consumed in the study chow (see Table 3.2), assuming average daily consumption of water and chow. These mice were then switched to normal water and a Western diet, in which the sucrose was replaced by D-tagatose and with dihydromyricetin added (TD. 140145), for the remainder of the study.

TD.140143, TD.140144, and TD.140145 were custom formulations by Harlan Teklad.

Table 3.1[49]

Comparisons of the 5 Diets Fed to the ApoE$^{-/-}$ Mice

Group	Diet	Sucrose % by weight	Cholesterol	D-tagatose % by weight	Fat % by weight	Dihydromyricetin	Kcal/g
1	Standard TD.2108	0	0	0	6.2%	0	3.1
2	Western TD.88137	34%	1.5 g/Kg	0	21%	0	4.5
3	D-tagatose TD.140143	0%	1.5 g/Kg	34%	21%	0	3.7
4	BSN723 TD.140144	34%	1.5 g/Kg	0	21%	1.11 g/Kg	4.5
5	BSN723T TD.140145	0	1.5 g/Kg	34%	21%	1.11 g/Kg	3.7

Table 3.2[49]

D-tagatose Run-In Schedule

Day	D-tagatose (g) / 100 ml water
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

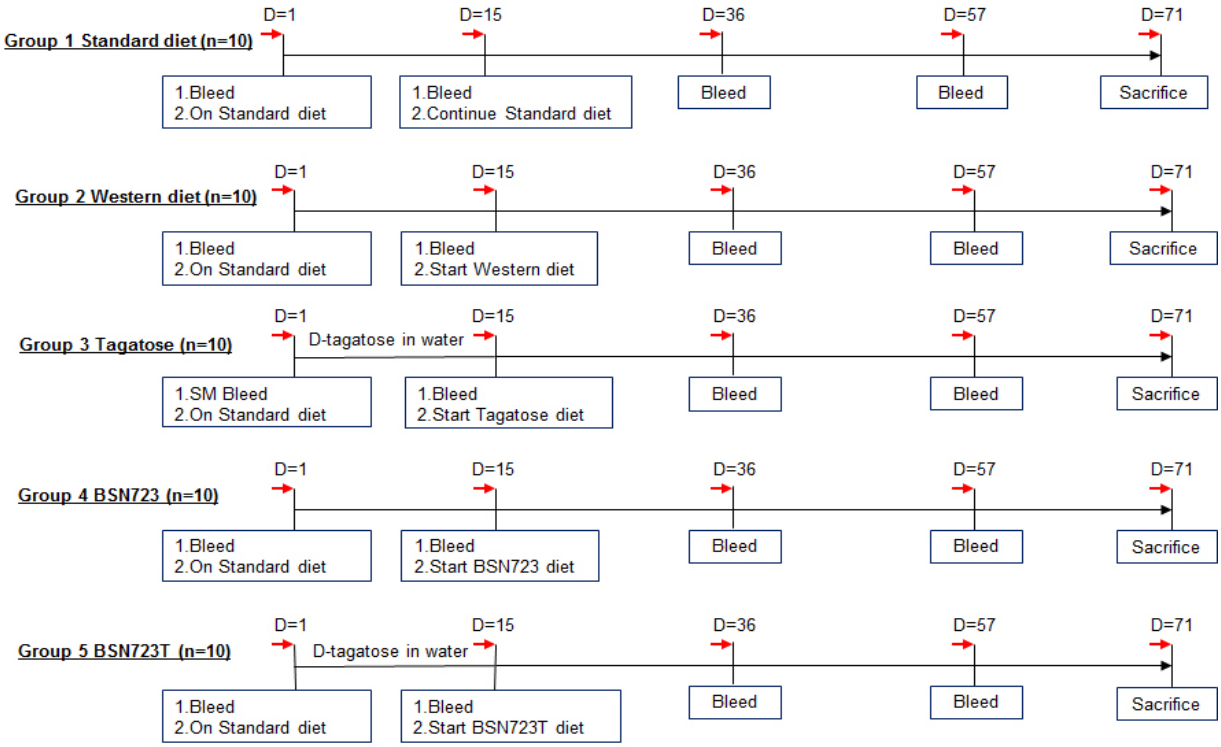
Addition of D-tagatose to drinking water during the two-week run-in phase. Increasing amounts of D-tagatose were added to drinking water to acclimate mice to D-tagatose. Mice in Groups 3 and 5 had D-tagatose added to their drinking water.[49]

Study Design

An outline of the time course of the study is shown in Figure 3.1.

Figure 3.1[49]

Outline of the Study Protocol



Due to the potential for gastrointestinal distress that results from the poor absorption of D-tagatose, ApoE$^{-/-}$ mice receiving D-tagatose in their diets (Groups 3 and 5) were acclimated to the sugar by adding increasing amounts of D-tagatose to their drinking water daily during a two-week run-in phase before beginning their respective diets (Table 3.2). D-tagatose was not placed in the chow for the tagatose run-in period because previous experience with powdered chow feeders had shown that much of the chow was lost in the litter and it was too difficult to weigh food consumption. Additionally, because there is a minimum order for custom chows, it would not have been cost effective to make pelleted chow for all of the different D-tagatose doses used in the run-in period.

After the D-tagatose run-in period, mice were maintained on their diets for 8 weeks. The diets consumed are shown in Table 3.1. 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 TD.140143 and TD.140145 chows, the sucrose has been replaced by D-tagatose. The ratios of calories from fat, protein and carbohydrates (fat/protein/carbohydrates) are as follows: TD.2108 (Standard) = 18/24/58; TD.88137 (Western) = 42/15/43; TD.140143 (D-tagatose) = 52/19/29; TD.140144 (BSN723) = 42/15/43; TD.140145 (D-tagatose + BSN723) = 52/19/29.

Body weight and food consumption was measured during the D-tagatose run-in phase and weekly thereafter during the dosing phase. Blood was sampled by submandibular bleeds after overnight fasts at multiple time points throughout the treatment period (see Figure 3.1), and total serum cholesterol and triglyceride levels were determined.

At the study end point, mice were euthanized using CO_2 followed by cervical dislocation, and the left ventricle was punctured to obtain blood. After the right atrium was cut, mice were exsanguinated by perfusion with saline through the left ventricle and tissues were removed (liver, kidneys, spleen, epididymal fat, retroperitoneal fat, subcutaneous fat, and aortic tissue from the heart to iliac bifurcation). The livers, kidneys, spleens and fat tissues were weighed. The hearts with the 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 serum cholesterol and total serum 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, in which 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. For determining the area of the aortic arch, a 3 mm line was drawn in software downward from the root of the left subclavian artery. Using the bottom of this line as a base, the intimal area of the arch was traced to determine the area. The atherosclerotic plaques were then traced and quantified using Nikon NIS Elements software.[78] Atherosclerotic lesions were quantified by two independent observers (labeled SG and JW). Data are expressed as the percentage of the aortic arch covered with grossly discernable atherosclerotic lesions.

Calculations and Statistics

Data are presented as the mean ± standard error of the mean (s.e.m.). 1-way ANOVA was utilized for analyses. Values of $p < 0.05$ were considered to be statistically significant.

3.4 Results[49]

One mouse in Group 1 died on day 42 after being bled. One mouse in Group 4 died on day 44 and was found on necropsy to have had encephalitis and meningitis of unknown origin. Data gathered from these two mice up until the time of their deaths were included in the analysis.

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 (Table 3.3). The mice on the Western and BSN723 diets consumed the highest number of calories per day (15.77 ± 1.13 Kcal/day and 16.34 ± 0.98 Kcal/day, respectively, the difference not being significant, $p = 0.69$) followed by the BSN723T (13.86 ± 0.98 Kcal/day), D-tagatose (11.65 ± 1.33 Kcal/day) and Standard diet (11.23 ± 0.49 Kcal/day) groups. The caloric intake between the following groups was statistically significant; Standard and Western diets (+, $p < 0.001$), Standard and BSN723 diet (+, $p < 0.001$), Standard and BSN723T diet (+, $p = 0.004$), Western and D-tagatose diet (*, $p < 0.001$), D-tagatose and BSN723 diet (*, $p < 0.001$) and the BSN723 and BSN723T diet groups (▲, $p = 0.014$). There was no significant difference in caloric intake between the Standard and D-tagatose, Western and BSN723, Western and BSN723T, or D-tagatose and BSN723T diet groups. There was no significant difference between the five groups in terms of food consumption by weight of chow eaten. One possible caveat regarding the estimated caloric values assigned to the chows containing D-tagatose is that the exact caloric value of the sugar is not a certainty and probably varies depending on the individual consuming it. Upon initial consumption of the sugar, it is poorly absorbed by the intestine, but absorption may increase over time with continued consumption. A value of 1.5 kcal/g of D-tagatose has been agreed upon for the use on food labels by the FDA and this is the value used to calculate the number of kcal/g in the chows containing D-tagatose.[3] Oxygen consumption measurements for each mouse in each study may be the best way to determine energy consumption in a particular study, but given the dependence of D-tagatose caloric content on the gut microbiome, such results may not be transferrable to other studies.

Table 3.3[49]

Comparison of food consumption and average caloric intake of mice according to diet

Diet	Grams of food/mouse/day	Kcal/gram chow	Kcal/mouse/day
Standard TD.2018	3.62 ± 0.16	3.1	11.23 ± 0.49
Western TD.88137	3.50 ± 0.23	4.5	15.77 ± 1.13, + *
TD.140143 (D-tagatose)	3.15 ± 0.36	3.7	11.65 ± 1.33
TD.140144 (BSN723)	3.63 ± 0.22	4.5	16.34 ± 0.98, + *
TD.140145 (BSN723T)	3.75 ± 0.26	3.7	13.86 ± 0.98, + ◆

Values are reported as mean ± s.e.m.[49]

\+ = significant compared to Standard group

* = significant compared to D-tagatose group

◆ = significant compared to BSN723 group

Body Weights

Mice in all five groups were on the Standard diet at the beginning of the study and during the two-week D-tagatose run-in period. At the end of the run-in period the body weights of the mice in all five groups were not significantly different (Figure 3.2A). On day 15 the mice were placed on their respective diets for 8 weeks.

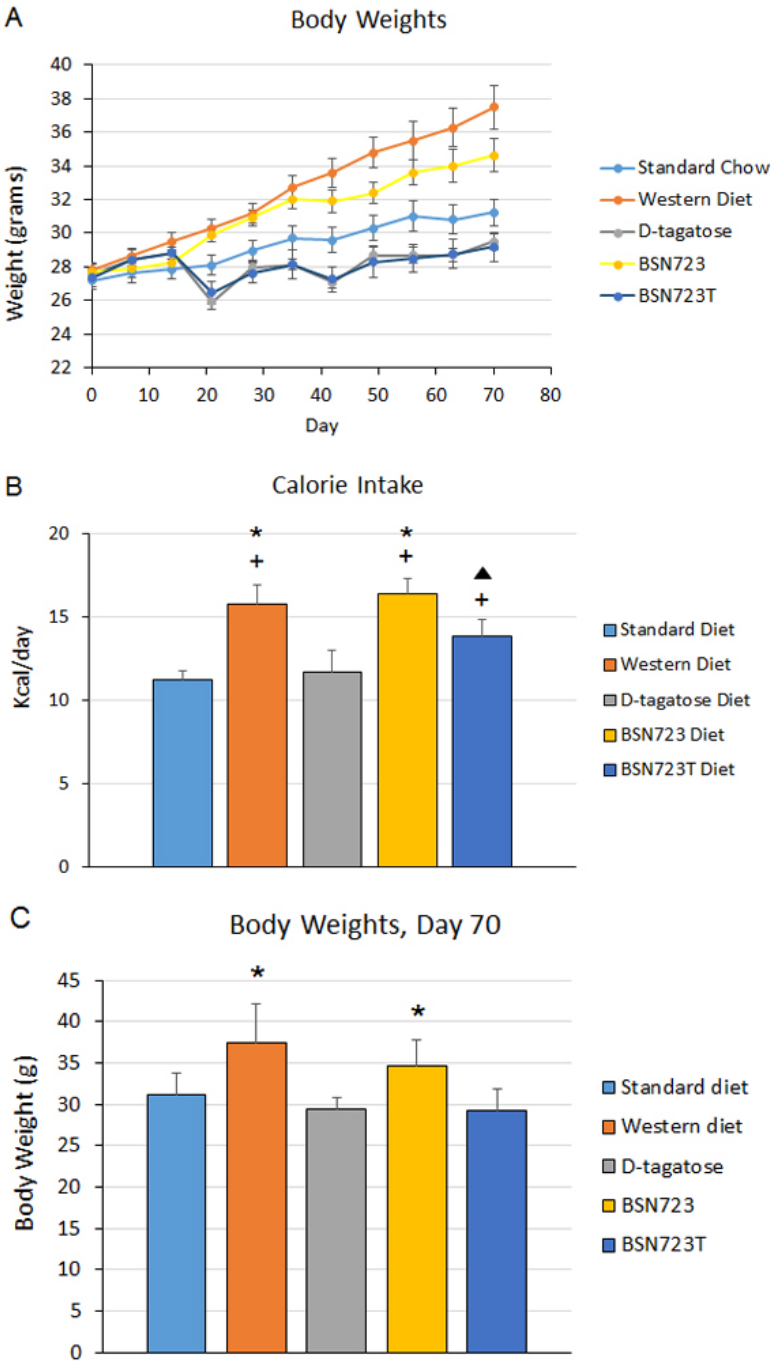
There was a drop in weight in the D-tagatose and BSN272T groups (Groups 3 and 5) between day 15, when the mice were started on their respective D-tagatose containing chows, and day 21 when the mice were next weighed. The mice in these two groups then gained weight at a rate comparable to the Standard diet mice and at the end of the study there was no significant difference between the weights of the mice in these three groups. Food consumption by mice in the D-tagatose and BSN272T groups was decreased compared to the other three groups during the first week the mice were placed on their respective diets and this is the likely explanation for the weight loss. After the first week, their food consumption increased and there was no significant difference between the five groups in terms of food consumption by weight of chow eaten. At the end of the study, mice on the Western diet weighed the most (37.5 ± 1.46 g) but not significantly more than mice on the BSN723 diet (34.6 ± 1.04 g, P = 0.13). Mice on the Western and BSN723 diets weighed significantly more (*, $p \leq 0.024$) than mice on the other three diets while there was no significant difference between the weights of mice on those three diets (Standard diet, 31.2 ± 0.89 g; D-tagatose diet, 29.5 ± 1.40 g; BSN723T diet, 29.2 ± 0.83 g). Differences in caloric intake between the 5 groups directly correlates with the final body weights of each group of mice and could account for any differences between the groups (Figure 3.2B).

Adipose Tissue and Organ Weights

Mice on the Western diet had increased total adipose tissue (epidydimal + retroperitoneal + subcutaneous adipose tissue) compared to mice on the Standard diet (Figure 3.3). The addition of D-tagatose, either alone or in combination with BSN723, prevented the increase in adipose tissue brought on by the Western diet and, in fact, mice consuming D-tagatose (Groups 3 and 5) were leaner than the mice on the Standard diet. Mice on the BSN723 diet were also leaner than those on the Western diet but were not significantly different from mice on the Standard diet. While mice on the BSN723 had more epididymal fat than mice on the Standard diet (0.67 ± 0.14 g vs 0.47 ± 0.09 g, respectively), the difference was not statistically significant ($p = 0.26$). The amount of subcutaneous (0.2 ± 0.03 g) and retroperitoneal fat (0.23 ± 0.06 g) in mice on the BSN723 mice was similar to that found in mice on the Standard diet (0.19 ± 0.04 g and 0.29 ± 0.07 g, respectively).

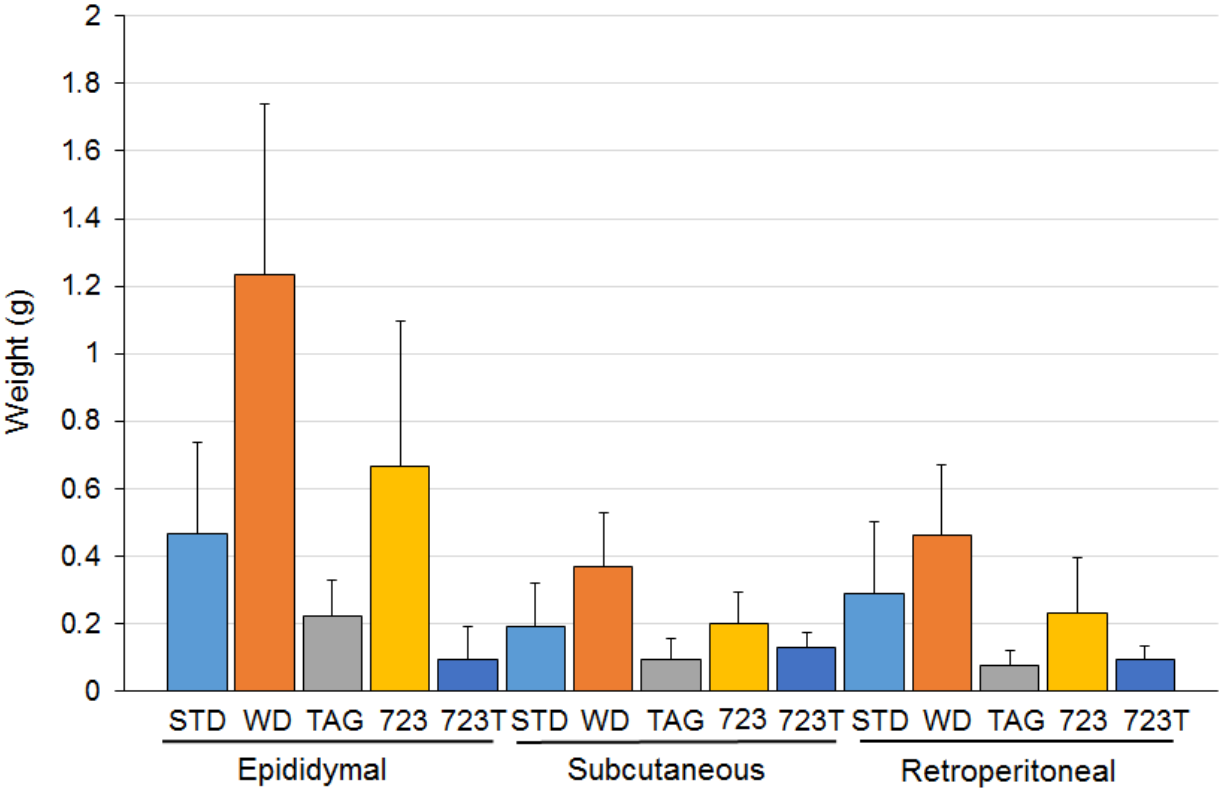
Figure 3.2[49]

Comparison of Body Weights and Caloric Intake Throughout the Study



All mice were kept on the Standard diet during the two-week D-tagatose run-in phase (days 0 to 14). Mice were then placed on their respective diets for eight weeks. (A) Change in body weights over time. (B) Caloric intake per group; * = greater compared to Standard, D-tagatose, and BSN723T groups; + = greater compared to Standard and D-tagatose groups; ▲= greater than D-tagatose group. (C) body weights at the end of the study; * = greater compared to Standard, D-tagatose, and BSN723T groups. Standard diet, n = 9-10; Western diet, n = 10; D-tagatose diet, n = 10; BSN723 diet, n = 9-10; BSN723T diet, n = 10. Results are reported as mean ± s.e.m.[49]

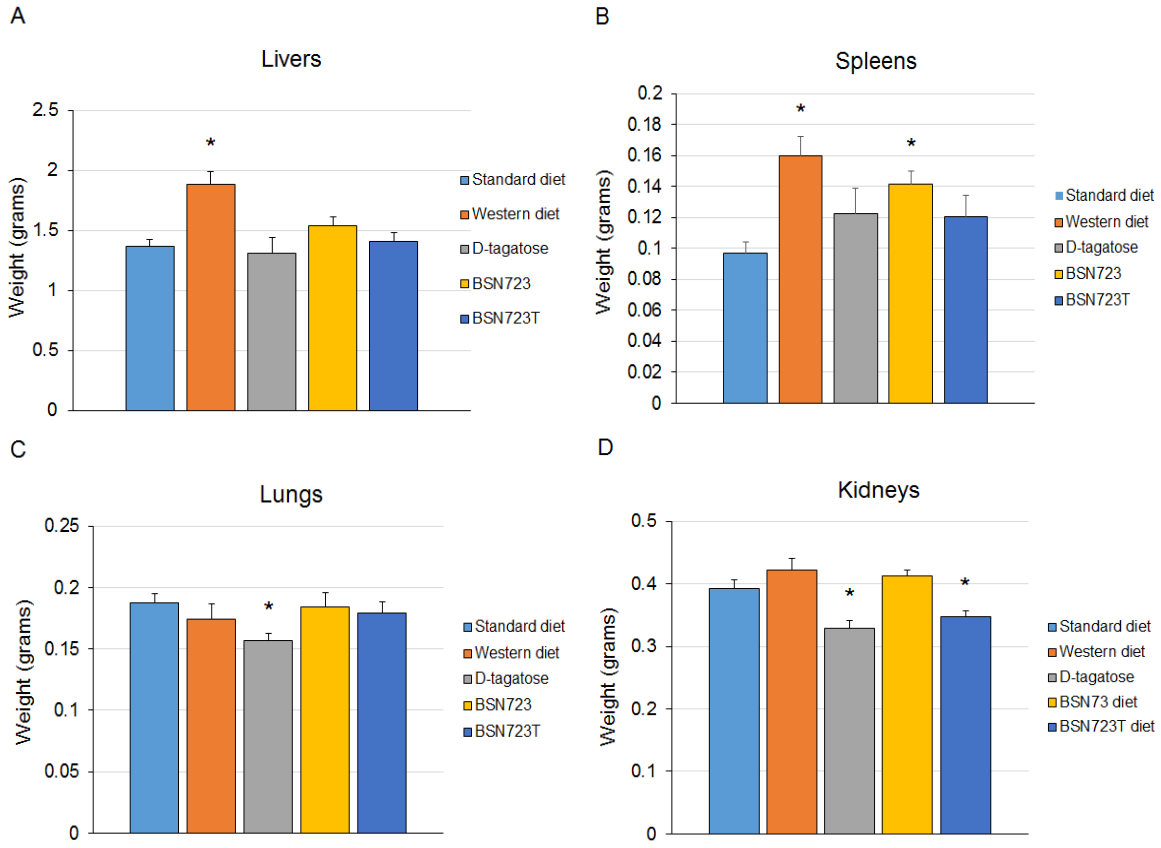
Figure 3.3[49]
Fat Pad Measurements



Adipose tissues from mice. Standard diet, n = 9; Western diet, n = 10; D-tagatose diet, n = 10; BSN723 diet, n = 9; BSN723T diet, n = 10. Results are reported as mean +/- s.e.m.[49]

Livers from mice on the Western diet weighed significantly more than livers from mice on all the other diets ($p < 0.02$) (Figure 3.4A). There was no difference between livers of mice from any of the other groups. The only significant differences in the weights of spleens were from mice on the Western and BSN723 diets compared to spleens from mice on the Standard diet ($p < 0.02$) (Figure 3.4B). There was no difference between spleens of mice from any of the other groups. The lungs from mice on the D-tagatose diet (Group 3) were slightly smaller ($p = 0.012$) than the lungs from the Standard diet fed mice (Figure 3.4C). There were no differences between any of the other groups. Kidneys from mice that received D-tagatose in their diets (groups 3 and 5) were slightly smaller than kidneys from mice in the other groups ($p < 0.02$) (Figure 3.4D).

Figure 3.4[49]
Measurement of Organ Weights

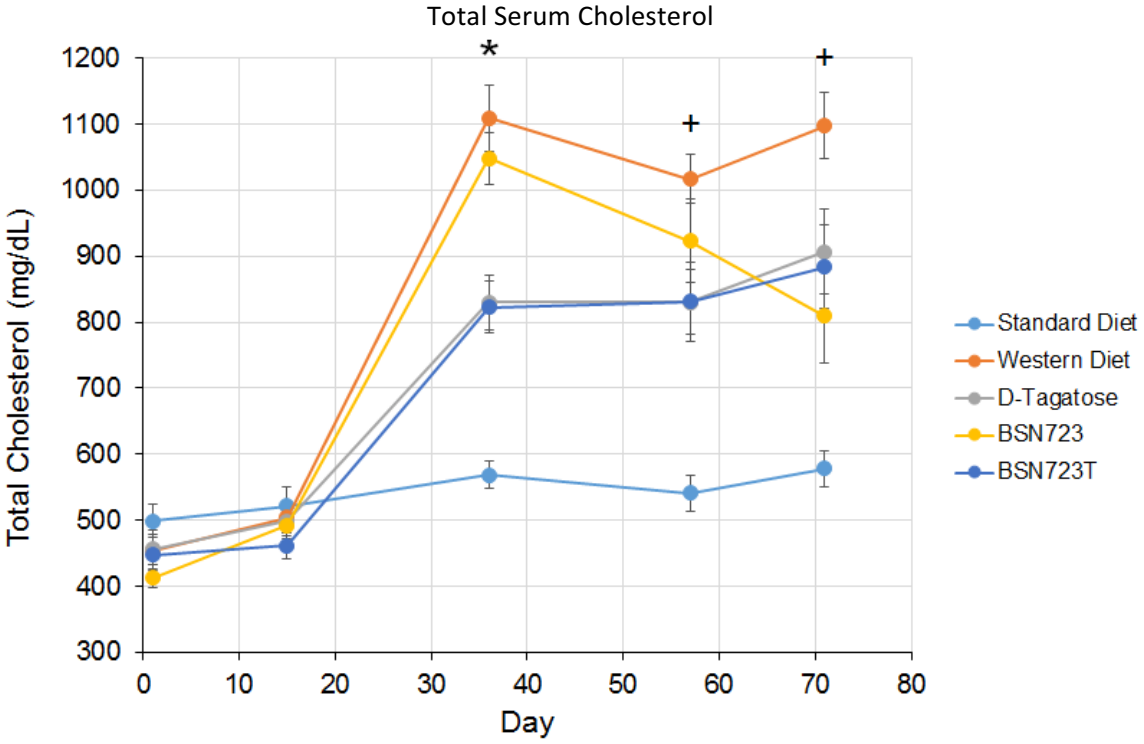


Organ weights. A) Livers. B) Spleens. C) Lungs. D) Kidneys. Standard diet, n = 9; Western diet, n = 10; D-tagatose diet, n = 10; BSN723 diet, n = 9; BSN723T diet, n = 10. Results are reported as mean +/- s.e.m.[49]

Total Cholesterol

During the D-tagatose 14-day run-in period all of the mice were on the Standard diet (day 1 to 14). At the end of the 14-day run-in period, there were no significant differences in serum total cholesterol levels between any of the groups (Figure 3.5). On day 15, the mice we started on their respective diets. By day 36, cholesterol had increased in the mice on the Western diet and the three treatment diets (Groups 3, 4, and 5) compared to mice on the Standard diet. However, the increase was significantly less in the two groups receiving D-tagatose (*, Group 3 and 5, $p < 0.0001$ for both groups) compared to mice on the Western diet. There was no significant difference in cholesterol between the mice on the Western and BSN723 diets on day 36. By Day 71 (end of study) total cholesterol in all three treatment groups was significantly less (+, D-tagatose, $p = 0.034$; BSN723, $p = 0.005$; and BSN723T, $p = 0.016$) than that of mice on the Western diet, but significantly higher than mice on the Standard diet (+, D-tagatose, $p = 0.0006$; BSN723, $p = 0.012$; and BSN723T, $p = 0.0007$).

Figure 3.5[49]



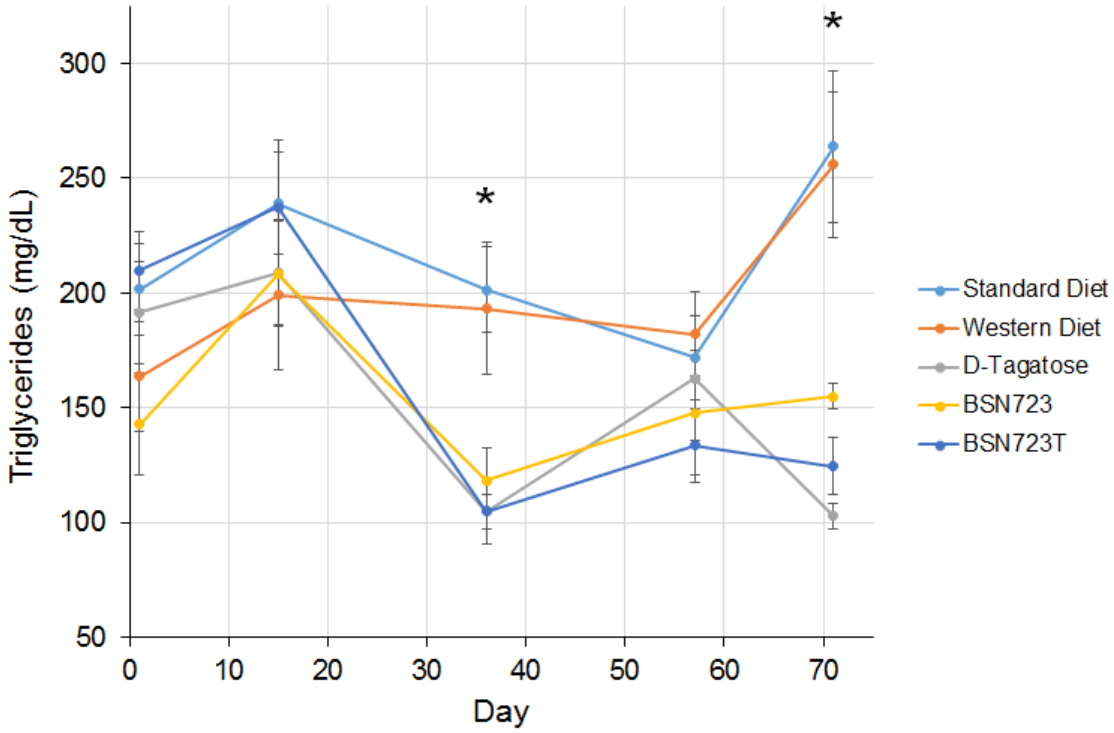
Time course of total serum cholesterol. All mice were fed Standard diet during the D-tagatose run-in (Days 1 to 14) and then placed on their respective diets. Standard diet, n = 9; Western diet, n = 10; D-tagatose diet, n = 10; BSN723 diet, n = 9; BSN723T diet, n = 10. Results are reported as mean +/- s.e.m. * = D-tagatose and BSN723T diet groups significantly different than Western and BSN723 diet groups. + = all treatment groups are significantly lower than Western diet group and higher than Standard diet group.[49]

BSN723 and D-tagatose Lower Serum Triglycerides Compared to Mice on Both the Standard and Western Diets

At the end of the 14-day run-in period, there were no significant differences in triglycerides between any of the groups (Figure 3.6). There was also no significant difference in triglyceride levels in mice on the Standard or Western diets at any time point during the study. The D-tagatose, BSN723, and BSN723T diets lowered triglycerides compared to mice on the Standard and Western diets during the course of the study. By day 71, triglycerides in Groups 3, 4, and 5 were approximately half or better than the levels in either the Standard or Western diet groups (Standard diet, 263.7 ± 33.1 mg/dL; Western diet, 256.0 ± 31.9 mg/dL; D-tagatose, 102.9 ± 5.65 mg/dL; BSN723, 155.0 ± 15.1 mg/dL; BSN723T, 124.7 ± 12.3 mg/dL; *, $p \leq 0.014$ Groups 3, 4, and 5 compared to Groups 1 and 2.) On Day 71, triglycerides were also significantly lower in the D-tagatose group than in the BSN723 group ($p = 0.009$).

Figure 3.6[49]

Serum Triglycerides



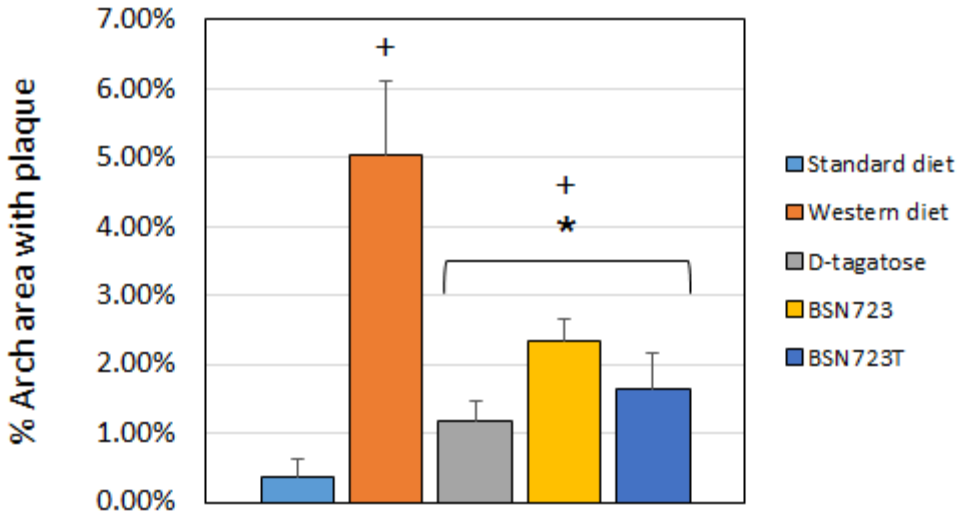
All mice were on the Standard diet during the two-week D-tagatose run-in period (Days 1 to 14) and then placed on their respective diets. Standard diet, n = 9; Western diet, n = 10; D-tagatose diet, n = 10; BSN723 diet, n = 9; BSN723T diet, n = 10. Results are reported as mean +/- s.e.m. * = All treatment groups significantly lower than Standard diet and Western diet groups. On day 71 D-tagatose and BSN723 groups were also significantly different.[49]

Atherosclerosis

The surface area of aortas covered by atherosclerotic lesion was greater in mice on the Western diet compared to mice on the Standard diet ($p = 0.001$) (Figure 3.7). The addition of D-tagatose, BSN723, or BSB723T to the diets inhibited the formation of plaque in aortas compared to mice on the Western diet ($p = 0.007$, $p = 0.04$, $p = 0.016$, respectively). The aortas from mice that were on the D-tagatose, BSN723, and BSN723T diets all had greater plaque formation compared to mice on the Standard diet ($p = 0.023$, $p < 0.0001$, $p = 0.02$, respectively). Significant differences were found between mice from Group 1 (Standard diet) and all other groups (+, $p < 0.022$), between Group 2 (Western diet) and all other groups (*, $p < 0.04$), and between Groups 3 (D-tagatose diet) and 4 (BSN723 diet) ($p = 0.014$).

Figure 3.7[49]

Atherosclerotic Plaque Coverage in the Aortic Arch



Comparison of the percent area of the aortic arch that has plaque. Aortas were prepared for atherosclerosis measurements via en face presentation. The addition of D-tagatose, dihydromyricetin or BSN723T to the Western diet significantly prevented the formation of atherosclerotic plaques. Standard diet, n = 9; Western diet, n = 10; D-tagatose diet, n = 10, BSN723, n = 9, BSN723T, n = 10. Results are shown as mean +/- s.e.m. + = significantly greater than Standard diet. * = significantly lower than Western diet group. The D-tagatose and BSN723 groups were also significantly different.[49]

3.5 Discussion[49]

Flavonoids are derivatives of 2-phenyl-1-benzopyran-4-1 and are present in fruits, vegetables, nuts, and seeds. Many studies have found an association between flavonoid intake and a reduction of risk for coronary events.[100–102] The flavonoid dihydromyricetin is the major bioactive compound in *Ampelsis grossedentata*, making up 15-20% (wt/wt) of the total dry weight of stems and leaves.[103] Many of the preliminary studies touting the positive effects of dihydromyricetin have utilized plant extracts which, in addition to containing high concentrations of dihydromyricetin, also contained complex mixtures of other flavonoids. Chen *et al*.[68] reported that the intragastric administration of extract from *A. grossedentata* to rats reduced serum total cholesterol and triglycerides and increased high-density lipoprotein. In a study using human subjects with hyperlipidemia, the administration of a drink made from *A. grossedentata* for 45 days reduced serum triglycerides, total cholesterol, and plasma lipids.[48] Up to now few studies have evaluated the serum lipid altering and anti-atherosclerotic activities of purified dihydromyricetin.

Considerable attention has been focused on the antioxidant activity of dihydromyricetin.[104,105] Liao *et al*.[105] crystallized dihydromyricetin from A. grossedentata and demonstrated it was effective at inhibiting the production of reactive oxygen species in treated cells thereby attenuating plasma lipid peroxidation. It also inhibited AAPH-induced production of malondialdehyde, an indicator of lipid peroxidation and a biomarker for oxidative stress. Evidence suggests that oxidative stress can lead to endothelial dysfunction and is involved in the development of atherosclerotic plaque.[106]

To our knowledge this is the first study that combines D-tagatose and BSN723 (dihydromyricetin) for the treatment of hyperlipidemia and the prevention of atherosclerosis. This study examined the effect of the administration of BSN723, D-tagatose, or a combination of the two, on weight

gain, blood lipids, and the development of atherosclerosis, in ApoE$^{-/-}$ mice consuming a Western diet (high fat, high cholesterol, high sucrose diet). In the diets containing D-tagatose, the D-tagatose replaced the sucrose in the Western diet formulation. For the mice treated with BSN723 alone (Group 4), sucrose was still present in the diet, BSN723 was just added to the chow formulation. The Western diet promoted obesity, increased total serum cholesterol, and markedly stimulated the development of atherosclerosis in the ApoE$^{-/-}$ mice compared to mice on Standard chow. In contrast, a diet that replaced gram-for-gram the sucrose in the Western diet with D-tagatose, with or without BSN723, prevented the weight gain seen with animals on the Western diet and had a marked effect on blood lipids and the development of atherosclerosis.

The mice on the D-tagatose diets 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$^{-/-}$ mice prevented the development of obesity.[78] We obtained the same result with ApoE$^{-/-}$ mice. Differences in caloric intake between the 5 groups directly correlates with the final body weights of each group of mice and could account for any differences between the groups. While not significantly different, mice on the diet containing BSN723 weighed less than mice on the Western diet (34.6 ± 1.04 g compared to 37.5 ± 1.46 g, respectively) even though the caloric intake of the BSN723 group (16.34 ± 0.98 Kcal/mouse/ day) was slightly, but not significantly, greater than mice on the Western diet (15.77 ± 1.13 Kcal/mouse/day).

There was a significant ($p < 0.01$) drop in weight in the two groups (Groups 3 and 5) on the diets containing D-tagatose between day 15, when the mice were started on their respective diets 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 the two groups on D-tagatose and the group on the Standard diet. 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 run-in period. While there were no outward symptoms of gastrointestinal distress in the mice placed on the D-tagatose containing diets, 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 on the Standard diet and the two D-tagatose containing diet groups (Groups 3 and 5), the mice on D-tagatose were leaner, as evidenced by the significantly lower amount of epididymal, retroperitoneal and subcutaneous fat compared to the Standard diet group. Similar results were seen in another study using LDLr$^{-/-}$ mice.[78] The addition of BSN723 to the Western diet (Group 4) resulted in mice that were leaner than those on the Western diet, even though the caloric intake of these two groups was essentially the same, but not as lean as mice on the Standard diet or the two D-tagatose containing diets.

While D-tagatose fed mice (Groups 3 and 5) exhibited increased serum cholesterol compared to Standard diet mice, the extent of these changes was far less than those observed in the mice on the Western diet (Figure 3.5). The livers of ApoE$^{-/-}$ mice are unable to remove circulating cholesterol efficiently and as a result these mice exhibit elevated serum cholesterol. Atherosclerotic lesion development is very dramatic in ApoE$^{-/-}$ mice fed a Western-type diet and the beginning stages of the disease can be found at 6 weeks.[97] With the ApoE$^{-/-}$ and LDLr$^{-/-}$ mouse models, dietary cholesterol, rather than the level of fat, exerts a major influence on the development of atherosclerosis.[107–109] 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.[43,108,110] Replacement of sucrose with D-tagatose in a Western diet was shown to decrease serum total cholesterol in LDLr$^{-/-}$ mice compared to mice on the Western diet with sucrose.[78] We obtained similar results with the ApoE$^{-/-}$ mice. Total cholesterol increased somewhat in the D-tagatose, BSN723, and BSN723T fed mice, upon placing them on their respective diets compared to Standard diet, but never reached the levels of the mice on the Western diet. In future work the effect of BSN723 on HDL and LDL cholesterol should be measured and compared to D-tagatose.

In ApoE$^{-/-}$ mice, there is no significant change in triglyceride levels when mice are placed on a Western diet compared to mice fed a Standard diet (Fig. 3.6). Serum triglyceride levels in ApoE$^{-/-}$ mice are elevated compared to wild-type mice, even when fed the Standard diet. The addition of D-tagatose, BSN723, or a combination of the two, lowered serum triglycerides in these mice back toward what would be considered normal levels (see negative control). D-tagatose alone appeared to be more effective at lowering triglycerides than BSN723 alone or the BSN723T combination.

The ApoE$^{-/-}$ mice in this study developed little atherosclerosis when maintained on the Standard diet, which does not contain sucrose or added cholesterol, and is lower in fat compared to the Western diet. All three modified diets, D-tagatose, BSN723, and BSN723T, reduced the amount of plaque formed in the aortas compared to mice on the Western diet (Figure 3.7).

One of the objectives of this study was to determine if the addition of BSN723 to the diet containing D-tagatose would have any additive or synergistic effect on blood lipids or atherosclerotic plaque formation. Addition of BSN723 to D-tagatose did not increase the efficacy for reducing cholesterol or atherosclerosis compared to D-tagatose alone. While no such effect was observed, BSN723 by itself did significantly prevent diet induced rises in blood cholesterol and the formation of atherosclerosis, with efficacy increasing over time in the latter part of the study. A longer study seems warranted. Administration of BSN723 with polydatin, which yielded a synergistic reduction of lipids with D-tagatose,[46] might also prove effective.

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Chapter 4 - BSN272 Prevents Western Diet-Induced Atherosclerosis and Excess Weight Gain in ApoE$^{-/-}$ Mice[111]

4.1 Preface

Although BSN723T was efficacious in preventing diet induced increases in total cholesterol and formation of atherosclerosis, it did not have activity towards triglycerides (TG) as was the goal. However, it did provide a comparator for the BSN272 study (Appendix C) and studies to come. Metts *et al.*[46] followed that BSN272 LDLr$^{-/-}$ study by conducting an 8-week study in ApoE$^{-/-}$ mice to study the Dynamic Data-Driven Application Simulation (DDDAS) developed from the previous LDLr$^{-/-}$ study (Appendix C) using uncorrelated doses by principal axis transformation. ApoE$^{-/-}$ mice were fed a standard diet, a Western diet, or a Western diet with the sucrose replaced with D-tagatose and 1 g of trans-polydatin added per kg of chow for 8 weeks.[46]

Treatment with BSN272 resulted in approximately 25-33% less development of atherosclerosis in every area of the dissected aortas that was measured compared to positive control group which was significant. Western diet mice had significantly greater fat pad mass than did BSN272 mice: epididymal fat 1.9 vs. 0.3g, retroperitoneal fat 0.6 vs. 0.03g, and subcutaneous fat 0.9 vs. 0.2g. Total serum cholesterol was significantly lower in the BSN272 group compared to positive control (930 vs. 630 mg/dl, p=0.025). Most importantly for our purposes, triglyceride levels were lower in the BSN272 group, although this result was not significant (141 vs. 110 mg/dl, p=0.16).[46]

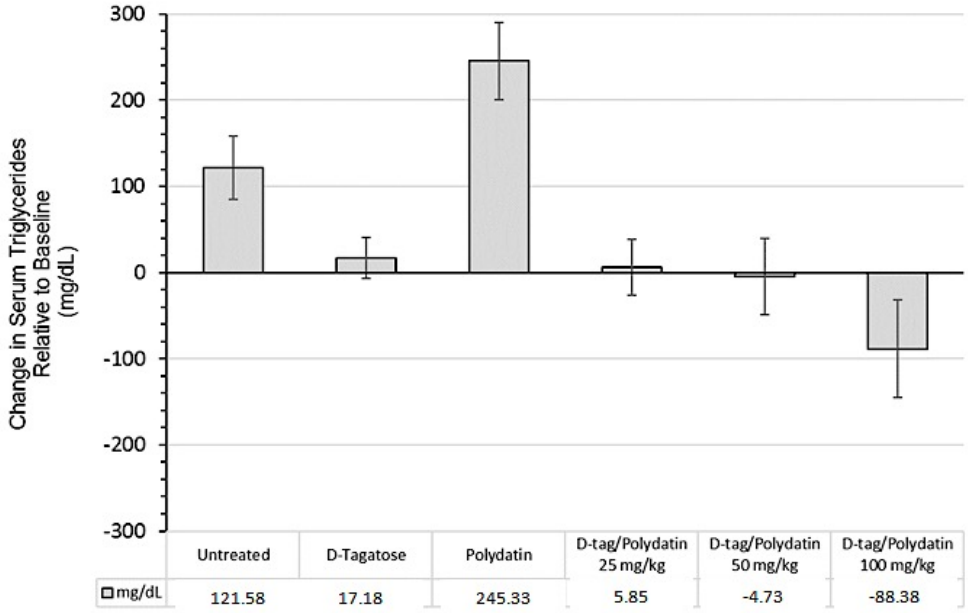
When the results of the study above were reintegrated into the DDDAS system and extrapolated it appeared as though the triglyceride results were not random and that a longer study with larger number of animals powered to obtain significance towards triglycerides would find a distinction between BSN272 treated mice and control.

This would be a substantial finding due to the fact that in the Phase III study D-tagatose led to an increase in triglycerides, and no study, to our knowledge, had shown the ability of D-tagatose to lower triglycerides (apart from one study by Police *et al*. that showed D-tagatose could prevent diet induced increases in triglycerides compared to positive control (sucrose/Western diet), although D-tagatose caused an increase in triglycerides compared to negative control (normal chow)).[78] In addition, an unpublished study in our lab using Syrian golden hamsters fed a high fat/carbohydrate Western diet (Untreated) showed that trans-polydatin actually led to increased triglycerides greater than the increase caused by Western Diet alone (Figure 4.1, compare untreated and polydatin columns). In the same study BSN272 using 100 mg/kg of the polydatin component was able to significantly reduce triglycerides from baseline (Figure 4.1, D-tag/polydatin 100 mg/kg column).

It should be noted that the increase in triglycerides from polydatin in our study is contrary to Arichi *et al*.,[112] Du *et al*.,[113] and Xing *et al*.[114] which all reported a reduction in TG with polydatin, which was partly responsible for its early selection for a combination with D-tagatose. In fact Du *et al*.,[113] using the same Syrian golden hamster model as in our study, reported the ability of polydatin to significantly prevent the diet induced increase in TG established with the high fat/cholesterol diet. A key difference between our studies is that the polydatin was initiated with the introduction of the high fat/cholesterol diet in the Du study, meaning that the study was actually designed to measure the ability of polydatin to prevent diet induced increases in atherosclerotic precursors. In our study, we allowed the hamsters to develop hyperlipidemia for 28 days prior to initiation of polydatin, and were therefore measuring the ability of polydatin to reverse hyperlipidemia. TG's in the Du study were around 16 mmol/L (142 mg/dL equivalent)[115] for the high fat/cholesterol group (positive control) at day 15 (study conclusion).[113] In our study

28 days after the initiation of the Western diet the serum TG average was 126 mg/dL in the positive control group. This is the point at which we began treatments for 21 days. During this time period the TG level in the Western diet group increased 122 mg/dL to a final end of study measurement of 245 mg/dl (change depicted in Figure 4.1). While polydatin was able to prevent the diet induced increase in serum TG's in the Du study,[113] it appears unable to reverse developed hypertriglyceridemia and in fact resulted in worsening TG levels that at the end of the study after 21 days of treatment with 100 mg/kg of polydatin averaged 359 mg/dL in the polydatin alone group.

Figure 4.1
Change in Serum Triglycerides



Graph depicts the change from day 1-21 (end of study) in serum triglycerides relative to baseline of each subject as an average ± SE. Hamsters were placed on a high fat/cholesterol/carbohydrate Western Diet on day -26 as those receiving D-tagatose began a D-tagatose run-in period until day 1 at which time all groups began the full study dose for 21 days. Polydatin alone group (100 mg/kg) received no treatment until day 1. (Unpublished study).

Regardless, the most important finding in our Syrian golden hamster study was that the combination of trans-polydatin and D-tagatose reversed developed hypertriglyceridemia more than could be expected from adding the results of the combo as monotherapy. TG's in the D-tag/polydatin 100 mg/kg group were reduced from 286 to 189 mg/dL throughout the course of the study (change depicted in Figure 4.1). This was especially intriguing because hamsters naturally have cholesterol ester transfer protein (CETP) as do humans and are considered to have a lipid metabolism profile similar to humans.[116]

There were complications in this study, the principal of which was that a miscalculation in the D-tagatose dose led to overdosing the D-tagatose groups for the first 11 days and resulted in the death of 12 hamsters from complications with diarrhea (likely due to the osmotic laxative effect of the carbohydrate load). The D-tagatose dose was subsequently cut in half for the remainder of the study but it's impossible to say what affect this may have had on D-tagatose administered groups. However, we had the original results from the $LDLr^{-/-}$ mice uncorrelated doses study that showed a trend towards lowering of TG's,[45] the follow-up study by Metts *et al*.[46] in $ApoE^{-/-}$ mice

that came just short of significance for lowering of TG's with the algorithm that predicted significance given a larger sample size, and now what appeared to be a robust result in diet induced hyperlipidemic hamsters with a questionable procedural mistake. It was time to perform a study with a large number of subjects over a lengthy period of time in the model of atherosclerosis and hyperlipidemia that we had had the most success with to determine definitively if BSN272 has efficacy against hyperlipidemia and atherosclerosis. In the study presented below, ApoE$^{-/-}$ mice were fed a Western diet and treated with BSN272 for 16 weeks. The figure and table numbers have been changed from the publication in order to be consistent for this dissertation.

4.2 Introduction[111]

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 diabetics, and is thought to be a major contributor to the increased incidence of cardiovascular disease seen in these two populations.[71,72] 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.[73] 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.[74]

Elevated serum cholesterol levels have been noted in rodents,[39] dogs,[40] nonhuman primates,[41] and humans[42] 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.[75] Low-density lipoprotein receptor deficient (LDLr$^{-/-}$) 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.[43]

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.[4,6,17] 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.[6] 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[29,30] leading to increased phosphorylation of glucose to glucose-6-phosphate leading to further activation of glycogen synthase.[31] There has been speculation that D-tagatose-1-phosphate can inhibit glycogen phosphorylase in the same manner that fructose-1-phosphate does,[6] 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,[76] 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.[4–6,13,18,37,58–60,77,78]

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, $LDL^{-/-}$ 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.[78] 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.[5] 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.[4,17]

There is considerable interest in the use of trans-resveratrol and its derivatives, including trans-polydatin, for the treatment of many human diseases.[117] 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.[118] Polydatin, otherwise known as trans-resveratrol or piceid, is a glucoside derivative of resveratrol and is the major component of these extracts. In addition to *Polygonum*, polydatin has been found in wines and grapes,[119–122] cocoa,[123] peanuts and peanut butter,[124] pistachios,[125] and almonds.[126] 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 possibly 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.[127,128] 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,[129,130] hepatoprotective,[131–134] anti-cancer,[135–138] neuroprotective,[129,139–141] and cardioprotective activities.[113,114,118,142,143] Pharmacological studies and clinical practice have demonstrated that polydatin also has protective effects against shock,[144,145] ischemia/reperfusion injury,[146,147] congestive heart failure,[148] endometriosis,[149] and prevention of fatty liver disease and insulin resistance,[150] and that it can regulate glucose and lipid metabolism.[151] Polydatin has found its way into clinical trials for the treatment of hemorrhagic shock and irritable bowel syndrome.[130,152]

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,[153,154] activation of protein kinase C,[155,156] suppression of NF-kappaB,[156] inhibition of the activation of renin-angiotensin-aldosterone system and decreasing the excretion of endothelin 1, TNF-α, and

angiotensin II,[142] reduction of lipid peroxidation levels,[127,157] up regulation of the expression of hippocampal brain-derived neurotrophic factor,[141] enhanced insulin sensitivity in the liver as shown by improved insulin receptor substrate 2 expression levels and Akt phosphorylation,[151] decreasing the content of malonydialdehyde (MDA),[140] 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,[154] restoring decreased deacetylase sirtuin1 activity and protein expression in liver tissue following severe shock[158] and activation of sirtuin,[159,160] suppressing oxidative stress-induced lysosomal instability and mitochondrial injury by increasing the protein expression of SOD2.[158]

The use of polydatin as a potential therapy for dyslipidemia has been suggested primarily by three studies using animal models.[112–114] Arichi *et al*.[112] 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.[113] In another study using rabbits, the administration of polydatin decreased the serum levels of total cholesterol, triglycerides, and LDL. The ratio of total cholesterol to HDL was also reduced.[114]

Insulin, through activation of the Akt pathway and other metabolic pathways, is a major component of metabolic regulation.[161] 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.[151] 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.[162] Additionally, polydatin is also thought to provide protection from oxidative peroxidation which can result in cell damage[127,163] and inhibition of oxidation of LDLs which may also play a role in atherosclerosis.[118]

In the present study, we have examined the effect of BSN272, a combination of D-tagatose and trans-polydatin, on blood lipids and atherosclerosis in ApoE$^{-/-}$ mice. The apoE lipoprotein resides on very low, intermediate, and high density lipoproteins (VLDL, IDL, 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.[97–99,164] 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.[98] We tested the hypothesis that BSN272 prevents Western diet induced elevations in serum triglycerides and cholesterol, and reduces the formation of atherosclerosis in ApoE$^{-/-}$ mice.

4.3 Methods[111]

Materials

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

Mice and Diets

Male Jax ApoE$^{-/-}$ 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 (Table 4.1).

Table 4.1[111]

D-tagatose Run-In Schedule

Day	D-tagatose (g) / 100 ml water
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

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.[111]

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 (341g D-tagatose/kg feed replacing 341g of sucrose, plus 1g 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 Table 4.2.

Table 4.2[111]
Comparisons of the Three Diets Fed to ApoE$^{-/-}$ 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

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 D-tagatose from near zero to something higher.

At the study end point, mice were euthanized using CO_2 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.[78]

Calculations and Statistics

Data are presented as the mean ± 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.

4.4 Results[111]

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 ± 0.63 Kcal/mouse/day) followed by the standard diet group (11.5 ± 0.87 Kcal/mouse/day) and then the BSN272 diet mice (10.08 ± 0.43 Kcal/mouse/day) (Table 4.3). 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 D-tagatose suggest that these energy results must be interpreted cautiously.

Table 4.3[111]

Comparison of Food Consumption and Average Caloric Intake of Mice According to Diet

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

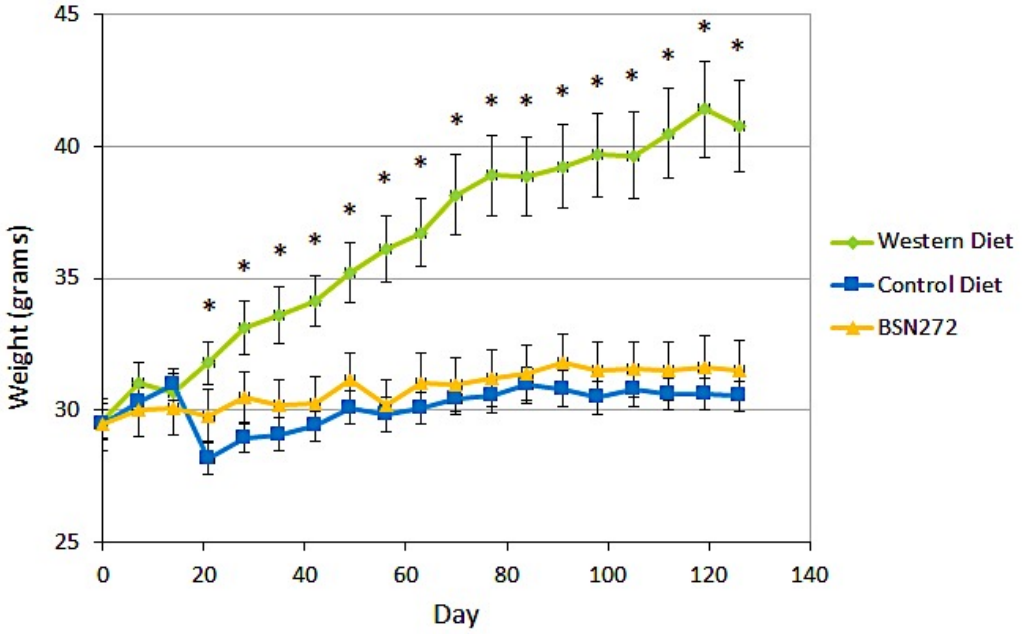
Food consumption was determined by weighing food given to the animals and food remaining after every 3 to 4 days. Results are shown as mean ± s.e.m. Standard diet, n = 9; Western diet, n = 13; BSN272 diet, n = 15.[111]

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 (Figure 4.2). 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 ± 1.72 g) at the end of the study than mice on standard (31.51 ± 1.11 g, $p < 0.01$) or BSN272 diets (30.53 ± 0.56 g, $p < 0.01$). There was no significant difference between mice in the standard diet and BSN272 groups ($p = 0.39$).

Figure 4.2[111]
Body Weights



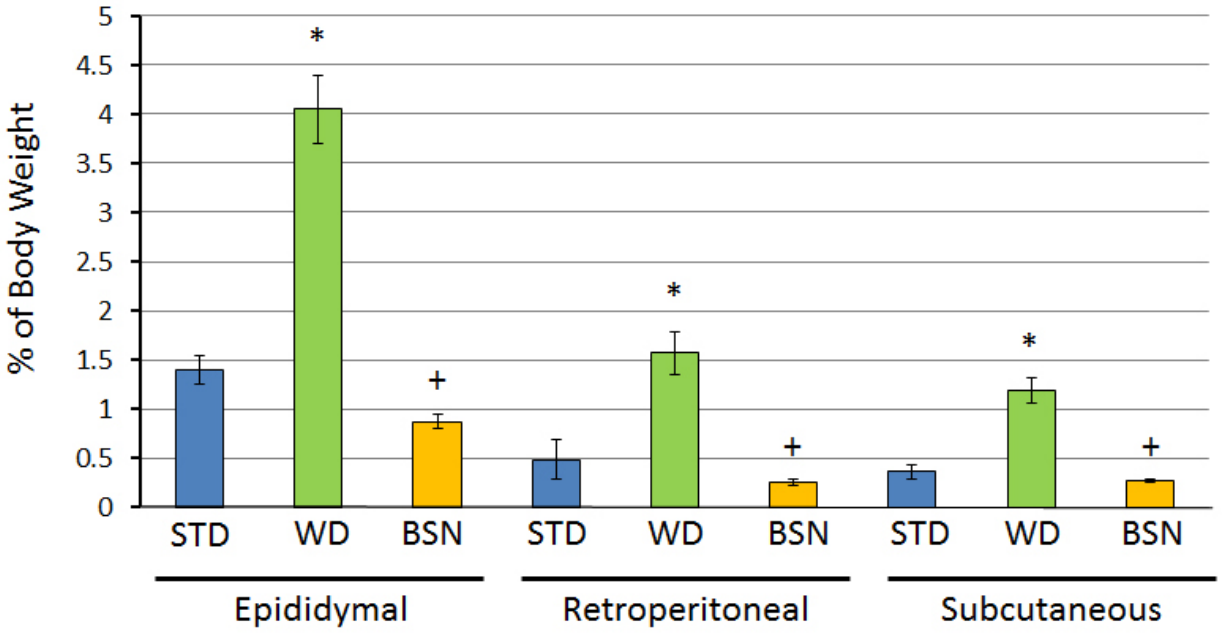
BSN272 prevented weight gain in mice associated with the Western diet. All mice were kept on the standard Diet during the two-week D-tagatose lead-in phase (days 0 to 14) during which mice in Group 3 were given increasing amounts of D-tagatose in their water until the concentration of D-tagatose resulted in equivalent consumption that would be obtained from the TD.110527 chow (BSN272). All mice were then placed on their respective diets for the remainder of the study. Results are shown as mean ± s.e.m. Standard diet, n = 9; Western diet, n = 13; BSN272 diet, n = 15. * $p < 0.01$ compared to both Standard and BSN272 diets.[111]

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 (Figure 4.3). 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 ($p < 0.01$).

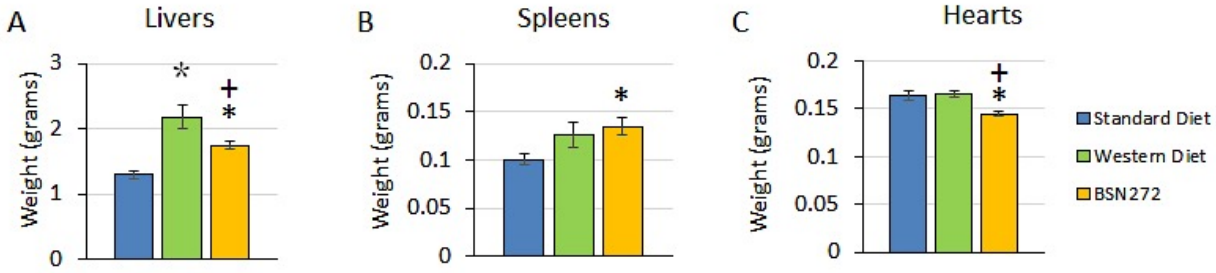
There was a significant increase in liver weights in mice on the Western (2.18 ± 0.19 g) and BSN272 diets (1.74 ± 0.061 g) compared to the Standard group (1.29 ± 0.054 g, $p < 0.01$) (Figure 4.4A). The BSN272 diet prevented some of the gain in liver size seen in the mice on the Western diet ($p < 0.05$). Spleens of the mice on the BSN272 diet were enlarged (0.135 ± 0.036 g) compared to spleens of mice on both the standard (0.100 ± 0.017 g) and Western diets (0.126 ± 0.046) (Figure 4.4B). 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*. 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.[59] Interestingly, in an unpublished 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 (Biospherics.net, unpublished study).

Figure 4.3[111]
Adipose Tissue Measurements



On day 127 fat pads were removed and weighed. Epididymal fat and retroperitoneal fat pads were removed from both sides of each animal and weighed, while subcutaneous fat was taken from one side only. Mice fed BSN272 were leaner compared to mice on the Western diet. (*, +, $p < 0.01$, comparisons between all groups). Results are shown as mean ± s.e.m. Standard diet, n = 9; Western diet, n = 13; BSN272 diet, n = 15.[111]

Figure 4.4[111]
Organ Weights



(A) Livers. *, $p = 0.001$, Westen vs Standard; +, $p < 0.001$, BSN vs Standard; *, $p = 0.027$, BSN vs Western. (B) Spleens. *, $p = 0.013$, BSN vs Standard. (C) Hearts. +, $p < 0.001$, BSN vs Western; *, $p = 0.047$, BSN vs Standard. Results are shown as mean ± s.e.m. Standard diet, n = 9; Western diet, n = 13; BSN272 diet, n = 15.[111]

Hearts from mice on the BSN272 diet weighed slightly less (0.144 ± 0.0137 g), than those from standard (0.164 ± 0.0297 g) or Western diet mice (0.166 ± 0.015) ($p < 0.01$ for Western vs. BSN272 and $p < 0.05$ for standard vs. BSN272) (Figure 4.3C).

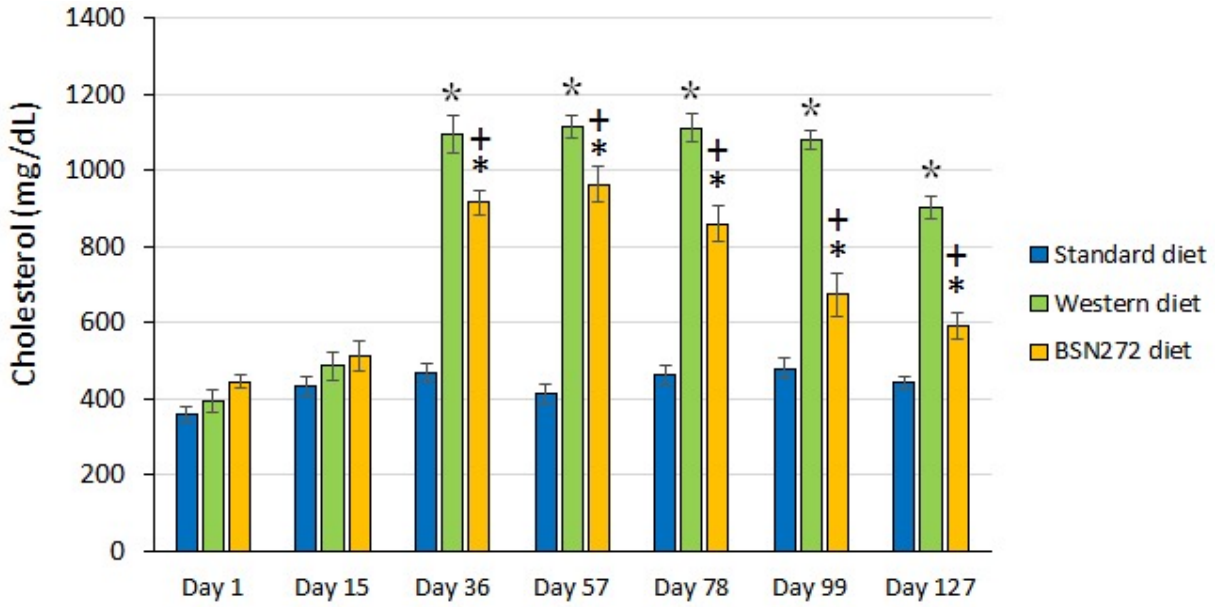
Serum Lipids

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 (Figure 4.5).

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 ± 35.6 mg/dL vs. 904 ± 28.1 mg/dL, $p < 0.01$).

Figure 4.5[111]
Total Serum Cholesterol



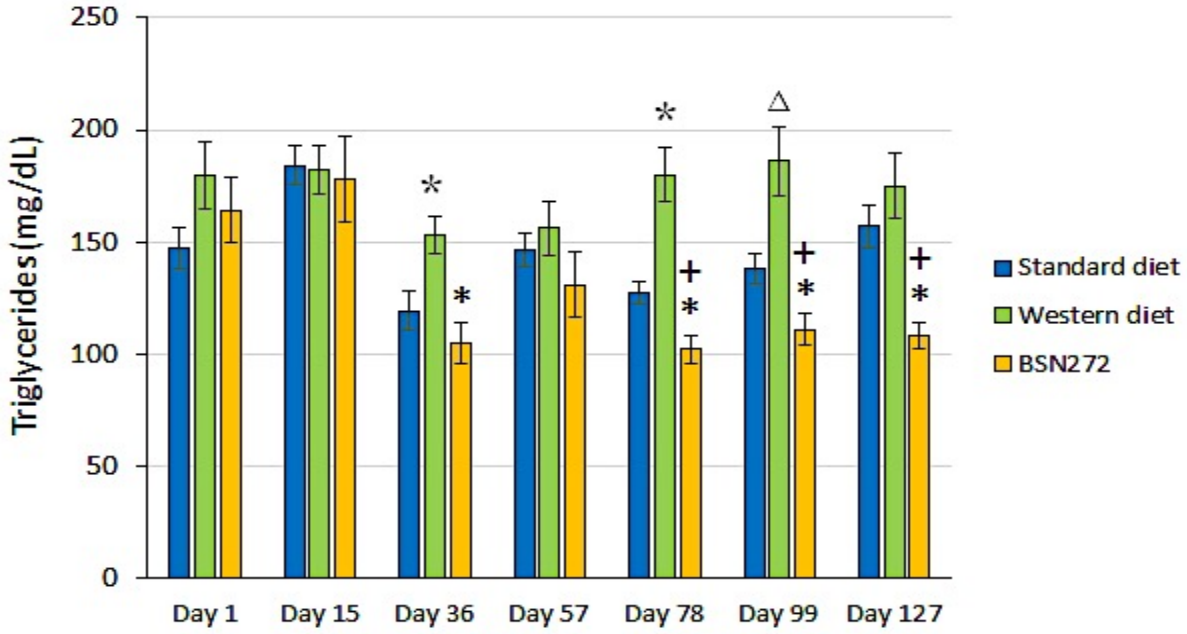
BSN272 blunts the rise in serum total cholesterol of mice fed a Western diet. All mice were on the standard diet during the two-week D-tagatose lead-in phase and then all groups were placed on their respective diets. Results are shown as mean ± s.e.m. Standard diet, n = 9; Western diet, n = 13; BSN272 diet, n = 15. *, $p < 0.02$ BSN272 vs Western diet; *, $p < 0.001$ compared to both Standard and BSN272 diets; +, $p < 0.02$ BSN272 vs Standard diet.[111]

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 (Figure 4.6). 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 ± 5.76 mg/dL) than mice on the standard diet (157 ± 9.51 mg/dL, $p < 0.01$) or the Western diet (175 ± 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.

Figure 4.6[111]

Serum Triglycerides



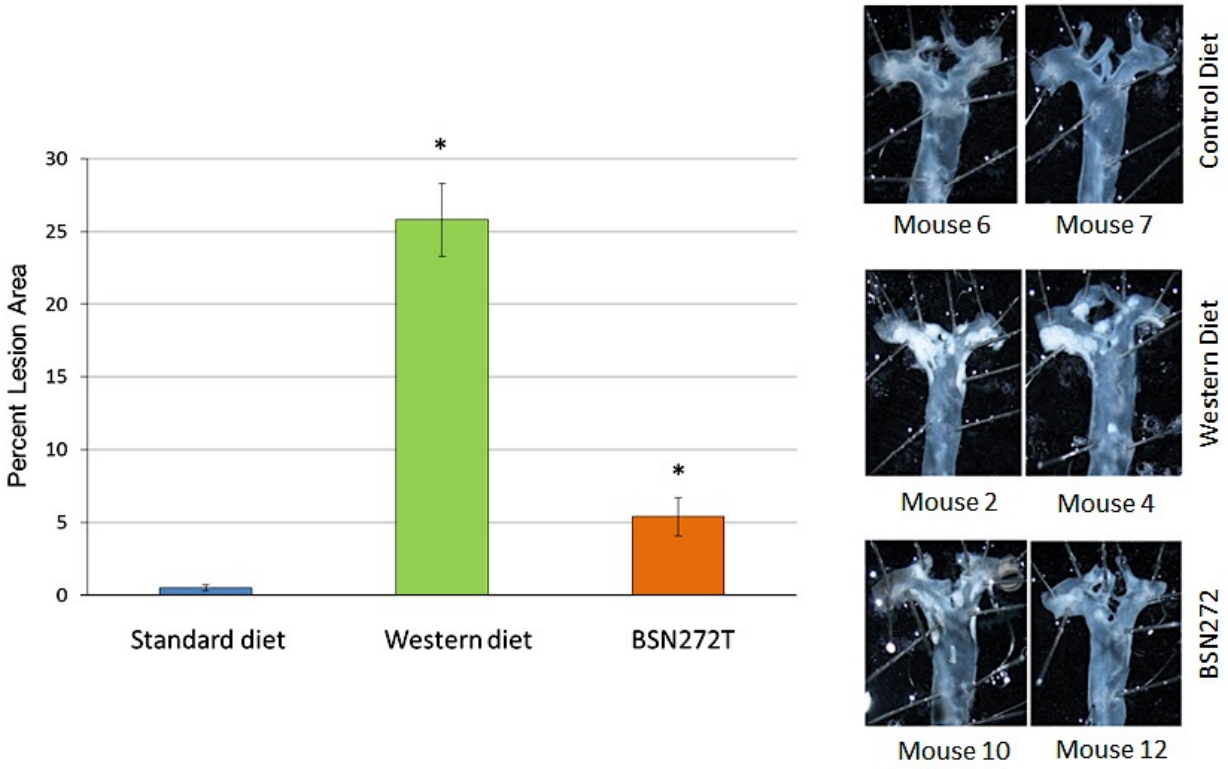
All mice were on the standard diet during the two-week D-tagatose lead-in phase and then placed on their respective diets. Mice fed BSN272 had lower serum triglyceride levels than mice on both the standard and Western diets. Results are shown as mean ± s.e.m. Standard diet, n = 9; Western diet, n = 13; BSN272 diet, n = 15. *, $p < 0.02$ BSN272 vs Western diet; *, $p < 0.02$ Western vs. Standard diet; +, $p < 0.02$ BSN272 vs Standard diet; Δ, $p < 0.03$ Western vs. Standard diet.[111]

Atherosclerosis

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 (Figure 4.7). 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 ± 2.51% compared to 5.30 ± 1.31% for mice treated with BSN272. Mice on the standard diet developed very little plaque (0.497 ± 0.195%), $p < 0.01$ for all comparisons.

Figure 4.7[111]
Plaque Formation in the Aortic Arch



Comparison of the area of the aortic arch that has plaque formation by percentage of measured area between the groups. Aortas were prepared for atherosclerosis measurements via en face presentation. The atherosclerotic plaques were then traced and quantified using Nikon NIS Elements software. The addition of BSN272 to the Western diet significantly prevented accumulation of plaque in the area of the aortic arch. Results are shown as mean ± s.e.m. Standard diet, n = 9; Western diet, n = 13; BSN272 diet, n = 15. *, $p <$ 0.001 between all groups.[111]

4.5 Discussion[111]

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 trans-polydatin. The high sucrose Western diet promoted obesity, increased total serum cholesterol and triglyceride concentrations, and markedly stimulated the development of atherosclerosis in ApoE$^{-/-}$ 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$^{-/-}$ mice prevented the development of obesity.[78] We obtained the same result with ApoE$^{-/-}$ 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$^{-/-}$ mice.[78] 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.[165,166] 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.[31,32] 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$^{-/-}$ 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$^{-/-}$ mice fed a Western-type diet and the beginning stages of the disease can be found at 6 weeks.[97] With the ApoE$^{-/-}$ and LDLr$^{-/-}$ mouse models, dietary cholesterol, rather than the level of fat, exerts a major influence on the development of atherosclerosis.[107–109] 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.[43,108,110] Replacement of sucrose with D-tagatose in a Western diet was shown to decrease serum total cholesterol in LDLr$^{-/-}$ mice compared to mice on the Western diet with sucrose.[78] We obtained similar results in this study with the ApoE$^{-/-}$ 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$^{-/-}$ mice were placed on the same BSN272 diet used here.[46] 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 ± 5.76 mg/dL) were significantly less than mice on the standard diet (157± 9.51 mg/dL, $p < 0.01$) or the Western diet (175 ± 14.86 mg/dL, $p < 0.01$). These triglyceride results mirror the earlier eight-week study[46] which, although not statistically significant, did result in reduced triglycerides in the BSN272 group (110.8 ± 62.5 mg/dL) compared to the Western diet group (141.7 ± 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 trans-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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Chapter 5 - Conclusions and Future Work

5.1 Preface

The 16-week BSN272 study in ApoE$^{-/-}$ mice and the toxicology study of trans-polydatin represented the last of the "First-in-Human (FIH)-enabling" nonclinical studies required by the FDA. The task then turned to packaging the various studies and data associated with D-tagatose, trans-polydatin, and BSN272, designing a phase 1 first in humans study, and submiting an indication with which to develop the IND towards. In this chapter is a summary of the IND submission, a presentation of the proposed Phase 1 PK study in healthy volunteers, and a discussion of Prader-Willi syndrome, the proposed indication for BSN272 for future work.

I spent the last 8 months packaging the IND submission. Even in a small company the recommended size for an "IND team" is a minimum of 3-4 people in order to properly package the submission and costs between $50-100,000 to outsource. While we collaborated as a lab team to write various sections of the submission, I compiled the entire submission, placed studies, descriptors, arguments, in the correct places, cross-linked the entire submission, and formatted it into the FDA's required structure. Each module was around 200 pages and the entire folder structure of documents, xml files, and pdfs was almost 2 GB in size.

A short overview of the IND application and the CTD structure is presented in section 5.2. The "Introduction to Summary" section of Module 2 is presented in section 5.3 to give an overview of the depth of studies conducted with BSN272 and its 2 components, as well as an introduction to Prader-Willi Syndrome. Section 5.4 provides an introduction to the proposed exploratory phase 1 PK microdose study of BSN272 in healthy subjects. The entire PK study proposal that will was included in the IND submission is available in Appendix E, with links disabled. Conclusions from the various studies and works presented in this dissertation are presented in section 5.5. Finally, a short look at the future work ahead can be viewed in section 5.6.

5.2 Overview

Investigational New Drug (IND) Application

In order to conduct clinical trials a new drug candidate must be approved by the FDA through the process of an individual new drug application. BSN272 was an unusually complex submission because it contained two molecules with plentiful studies conducted each as single agents, although the drug was treated as a single entity due to the synergistic nature in which the molecules work together. However, this required the submission of toxicity data for each molecule as well as efficacy in preclinical studies, and for the combination where appropriate. The toxicity profile needs to include the target organs and the types of toxicities to expect, the levels of drug exposures that cause these toxicities, the maximum tolerated dose, and the no-observed-adverse-effect-level (NOAEL). In addition to these concerns, as this is the first time trans-polydatin will be administered in the presence of D-tagatose in a clinical study, the FDA requested a micro-dosing PK protocol for the trans-polydatin component of BSN272.

Common Technical Documents (CTD)

Common Technical Documents (CTD) is the format the FDA requires the IND to be submitted in, in an effort to harmonize submissions across the US, Europe, and Japan. The CTD consists of 5 modules. Module 1 contains administrative information. Module 2 contains the summaries of Modules 3-5. Module 3 contains the quality information for manufacturing of the drug. Module 4 contains the non-clinical studies. Module 5 contains the clinical studies. Relevant information from Module 2 will be presented here as a summary of the IND submission. The CTD grows as

studies are completed on its way to a New Drug Application (NDA) and areas are filled in more thoroughly over time. The FIH filing must contain at least one safety/efficacy study in 2 separate mammalian models as well as a toxicology study, in this case one for D-tagatose and trans-polydatin each. The drug substance must also have a material safety data sheet (MSDS) from the manufacturer and proof of good manufacturing practices (GLP). Section 5.3 below should be used as a reference to understand what went into the BSN272 submission and where the drug development process is headed. This is the Introduction to Summary section that has been cut of Module 2 from the IND submission.

5.3 Module 2 (CTD Summaries) - Introduction to Summary

Biospherics.net LLC is developing the new combination drug product BSN272, composed of D-tagatose, also known as D-lyxo-hexulose ($C_6H_{12}O_6$, CAS RN 17598811), and trans-polydatin, also known as polydatin or piceid ($C_{20}H_{22}O_8$, CAS RN 27208-80-6), for the treatment of dyslipidemia, diabetes, satiety, and obesity in Prader-Willi Syndrome (PWS). Biospherics.net intends to conduct an exploratory Phase 1 pharmacokinetic (PK) microdose study to determine if the presence of D-tagatose affects the PK profile of polydatin before filing a complete IND application for BSN272.

Studies have shown that persons with Prader-Willi Syndrome (PWS) are at risk of premature death. PWS is the most commonly known genetic cause of life-threatening obesity (estimated prevalence of 1:15,000), resulting from obesity-related problems such as respiratory failure and pulmonary hypertension, obstructive sleep apnea, hypertension, cardiovascular disease, and type 2 diabetes mellitus (T2DM). The cause of death in adults is usually related to failures of the circulatory or respiratory systems. There is currently no single drug available that can successfully treat all of the symptoms of this complex disorder.

The major medical concern for patients with PWS is morbid obesity. Compulsive eating and obsession with food usually begins between the ages of 1 and 6. The urge to eat is physiological and overwhelming; it is difficult to control and requires constant vigilance. Weight control depends on food restriction and daily exercise. Currently there is no medication or surgical intervention that can eliminate the need for strict dieting and supervision around food. Studies have repeatedly shown that PWS patients are at risk of premature death. Mortality in children is most commonly associated with respiratory infection and high temperature resulting in sudden death. Common in adults with PWS are obesity-related problems such as respiratory failure and pulmonary hypertension, obstructive sleep apnea, hypertension, and type 2 diabetes mellitus (T2DM).[167–170] A high incidence of alterations in glucose metabolism including impaired fasting glucose, impaired glucose tolerance, and T2DM, has been observed in patients with PWS, particularly after adolescence.[171] Mean age at onset of diabetes is about 20. A prevalence rate of 20-25% for T2DM is found in patients with long standing PWS.[171,172]

Obesity and diabetes are well-established risk factors for premature cardiovascular disease. A limited number of studies have looked at cardiovascular fitness and disease markers in PWS patients. Cardiovascular risk factors are evident in a large percentage of children with PWS. de Lind van Wijngaarden *et al*. (2010) looked at the cardiovascular and metabolic risk profile in 85 children with PWS. They found that infants and prepubertal children had at least one cardiovascular risk factor. In 63% of the infants and 73% of prepubescent children, at least one of the following factors was present: elevated systolic or diastolic blood pressure; elevated serum total cholesterol, low density lipoprotein cholesterol, triglycerides, or lipoprotein(a) levels; or reduced high density lipoprotein cholesterol levels, indicating an unfavorable cardiovascular profile in addition to a high fat%, indicating an unfavorable cardiovascular profile.[173] Patel, *et al*. (2007) assessed cardiac and vascular structure and function in nine PWS patients and found

significantly elevated high-sensitivity C-reactive protein (hs-CRP), hs-CRP has been shown to be significantly elevated in patients dying suddenly with severe coronary artery disease,[174] and evidence of microcirculatory dysfunction as evidenced by decreased peak hyperaemic flow response.[175] In a small study involving 36 adults with PWS who were followed for 10 years there were 10 deaths.[176] The average age at death was 33.2 years with 60% of the deaths due to cardiovascular reasons including coronary occlusion, stroke, or heart failure. Lionti, *et al*. (2012) also concluded that cardiac or respiratory conditions were common causes of death of those with PWS after the age of 15 years.[168]

D-tagatose (D-lyxo-hexulose) is an enantiomer of D-fructose and occurs naturally in small amounts in dairy products. Heat treatment causes D-tagatose to form from galactose by isomerization. D-tagatose is approved as a safe food ingredient (Joint FAO/WHO Expert Committee on Food Additives (JECFA), 61st JECFA (2003), published in FNP 52 Add11 (2003))[177] and has been determined Generally Recognized As Safe under the intended conditions of use in foods by the FDA (GRAS; GRN 000078[2] and 352[3]). Extensive human studies have been completed showing the safety of D-tagatose for use in foods. A number of clinical trials demonstrating the potential antidiabetic activity of D-tagatose were conducted on healthy subjects and patients with type 2 diabetes mellitus (T2DM) without an IND by considering D-tagatose as a food additive. These trials demonstrated that D-tagatose did not raise postprandial blood glucose and blunted the rise in blood glucose when taken with meals.[2,3] In November 2005, after review of pharmacological, toxicological, and efficacy data from completed Phase 1 and 2 trials, the FDA/CDER activated an IND (# 70971) for D-tagatose and found it to be qualified for a Phase 3 trial for the treatment of T2DM, largely because of its safety profile. D-tagatose has since been clinically tested in one Phase 3 safety/efficacy and one Phase 2 dose ranging trial.[18,37] D-tagatose has been safely administered to humans up to 0.64 g/day, although some consumers have experienced mild and transient gastrointestinal tolerance such as diarrhea, flatulence, and abnormal gastrointestinal sounds at that dose.

The exact mechanism of action by which D-tagatose blunts the glycemic response to a meal is not entirely understood. However, based on the results of a number of studies with D-fructose and D-tagatose, a plausible mechanism of actions for the use of D-tagatose for glycemic control has been proposed. D-tagatose is metabolized in the liver in an identical manner to that of D-fructose. D-tagatose is phosphorylated to D-tagatose-1-phosphate by fructokinase, which is further metabolized by aldolase to yield glyceraldehyde (GA) and dihydroxy-acetone phosphate (DHAP). Although aldolase acts on both D-fructose-1-P and D-tagatose-1-P, the cleavage of D-tagatose-1-P occurs at only about half the rate of that of D-fructose-1-P.[32,33] Like D-fructose-1-P, the increase of D-tagatose-1-P concentration stimulates glucokinase activity[29,30,178] leading to an increased phosphorylation of glucose to glucose-6-P, which further activates glycogen synthase.[31] In addition, a literature review also suggests that D-tagatose-1-P, in a manner similar to D-fructose 1-P, has the ability to inhibit glycogen phosphorylase.[179,180] The net effect of the regulation of these two enzymes is an increase in glycogen synthesis and storage and a decrease in glycogen utilization. In addition to its effect on glycogen regulation, D-tagatose-1-P is capable of inhibiting sucrase activity leading to the suppression of sucrose digestion in the small intestine.[2]

At least one unconfirmed study, also found D-tagatose to inhibit maltase activity, and, thus, to delay the digestion of starch.[2] Thus, it seems plausible that D-tagatose is capable of depressing the hyperglycemic response to a meal or to a glucose load by several mechanisms including increasing glycogen synthesis and storage, decreasing glycogen utilization, and, possibly, also by

reducing the digestion of sucrose and other carbohydrates in the small intestine. The net effect is a reduction of glycemic levels and HbA1c closer to normal, healthy levels.

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, $LDL^{-/-}$ 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.[78] 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.[37] 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 2 diabetics taking D-tagatose showed a decrease in HbA1c and serum triglycerides.[4,5]

Trans-polydatin (t-PD or polydatin), a glucoside derivative of resveratrol, is purified from the roots of Polygonum cuspidatum and is found naturally in a number of foods including grapes, grape-containing products, cocoa, peanuts, peanut-containing products, pistachios, almonds, and hops. 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.[127,128] These properties would suggest that polydatin may have greater bioavailability than resveratrol.

There are many reported health benefits of trans-polydatin. Numerous studies have presented evidence that polydatin has positive health effects including anti-inflammatory,[129,130] hepatoprotective,[131–134] anti-cancer,[135–138] neuroprotective,[129,139–141] and activities deemed cardioprotective.[113,114,142,143] Additionally, trans-polydatin has shown through pharmacological studies and clinical practice to have protective effects against shock,[144,145,181] ischemia/reperfusion injury,[146,147] congestive heart failure,[148] endometriosis,[149] and prevention of fatty liver disease and insulin resistance,[150] and that it also may regulate glucose and lipid metabolism.[151] Polydatin has entered clinical trials for the treatment of hemorrhagic shock and irritable bowel syndrome.[130,152]

The mechanism for each of these outcomes is still being investigated. Some of the suggested mechanisms from evidence are: an antioxidant, free radical-elimination mechanism,[153,154] protein kinase C activation,[155,156] NF-kappaB suppression,[157] inhibiting of the activation of renin-angiotensin-aldosterone system which decreases the excretion of endothelin 1, TNF-α, and angiotensin II,[142] reduction of lipid peroxidation levels,[127,157] increased expression of hippocampal brain-derived neurotrophic factor,[141] enhanced insulin sensitivity in the liver as shown by improved insulin receptor substrate 2 expression levels and Akt phosphorylation,[151] decreasing the content of malonydialdehyde (MDA),[140] promoting activities of total superoxide dismutase (T-SOD), catalase and glutathione peroxidase (GSH-Px) in plasma, increasing glutathione (GSH) content in myocardial tissue,[154] decreasing deacetylase sirtuin1 activity and protein expression in liver tissue following severe shock[158] and thereby activating sirtuin,[159,160] suppressing oxidative stress-induced lysosomal instability and mitochondrial injury by increasing the protein expression of SOD2.[158]

The use of trans-polydatin as a potential therapy for dyslipidemia has been suggested principally by three studies using various animal models.[112–114] Arichi *et al*. discovered that orally administered trans-polydatin (100 mg/kg body weight) significantly lowered low-density lipoprotein (LDL)-derived cholesterol (by 18%) and serum triglycerides (by 40%) in rats consuming standard chow containing a mixture of corn oil, 10% cholesterol, and 1% cholic acid to induce increased blood lipids. Lower doses of trans-polydatin (50 mg/kg body weight) were ineffective at preventing hyperlipidemia, however, 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 lesser extent.[112] In a study using Syrian golden hamsters, polydatin was decreased total cholesterol (TC) levels and total triglyceride levels by 47% and 63%, respectively, when co-administered with a high fat/cholesterol diet and compared to standard diet.[113] In another study using rabbits, the administration of polydatin decreased total cholesterol in the serum as well as triglycerides and LDL.[114] The ratio of TC to HDL was also reduced.

Insulin is a major component of metabolic regulation through activation of the Akt pathway and other metabolic pathways.[161] Hao *et al*. recently found that trans-polydatin activated the Akt signaling pathway in diabetic rats. This was possibly accomplished by phosphorylation of the insulin receptor substrate (IRS), thereby reducing blood glucose levels.[151] Polydatin may also decrease the expression of intercellular adhesion molecule 1 (ICAM-1), reducing white blood cell adhesion, as well as the effects of other cell adhesion molecules and inflammatory cytokines. These are thought to be major components active in early atherosclerotic development.[162] Additionally, polydatin is thought to provide protection from cell damage resulting from oxidative peroxidation[127,163] and inhibition of oxidation of LDLs, which plays a large role in atherosclerosis.[118]

Polydatin has been administered to humans up to 85.5 mg/70 kg of body weight and no adverse events were reported.[182] To determine whether higher levels of trans-polydatin could be administered to humans, Biospherics.net evaluated the safety of polydatin (batch number BIPL110714) in a 28-day GLP rat toxicology study with a toxicokinetic test-arm and established a no observed adverse effect level (NOAEL) of 3.0 g/kg/day (the highest dose tested) in males and 1.2 g/kg/day in females. The 1.2 g/kg/day in females was based on bilateral moderate pelvic inflammation and transitional cell hyperplasia in the kidneys of one female given 3.0 g/kg/day.

The approach to the proposed initial Phase 1 Exploratory IND PK study is to orally administer a single dose of D-tagatose, trans-polydatin, and BSN272 to healthy males, collect blood samples prior to and after dosing, and quantify the serum levels of trans-polydatin and metabolites. The doses of D-tagatose and trans-polydatin when administered alone or in combination would not exceed 70 mg/kg and 100 ug respectively and the batch of trans-polydatin that will be used will be the same one that was tested in the 28-day rat toxicology study. The study medications will be provided in powdered form with no excipients to be mixed with 6 ounces of water for administration.

5.4 Exploratory Phase 1 PK Microdose Study in Healthy Volunteers

The primary objective of this phase 1 exploratory, microdose study is to determine whether the t_{max} and C_{max} of trans-polydatin (t-PD) change significantly when co-administered with D-lyxohexulose (DLH, or D-tagatose) in healthy male and female volunteers over a 24hour period. BSN272 is a potential drug therapy for the treatment of persons with Prader-Willi Syndrome (PWS). Healthy volunteers will be administered doses of trans-polydatin or BSN272. Blood samples will be taken pre-dose (t = 0) and at approximately 0.167 (10 min), 0.333 (20 min), 0.5, 1, 2, 4, 6, 8, 12 and 24-36 hours post drug administration. Urine samples will also be collected during the 12 hour onsite clinical period, with one subsequent sample collected at visit 3, which should

approximate the 24-hour posttreatment period. Serum and urine samples will be assayed for trans-polydatin and the metabolite trans-resveratrol at each time point to determine the pharmacokinetic effects of the combination BSN272 drug.

Healthy male and female volunteers, aged between 18 and 30 years old will be recruited by advertisement at the University of Kentucky. Acceptance into the trial will be based upon the full inclusion and exclusion criteria listed below. After giving informed consent, initial screening performed (physical exam, blood analysis including HIV and Hep C, and urinalysis) and when acceptability of the subject has been determined they will be invited to participate in the study. Subjects will be compensated for their time. Inclusion and exclusion criteria can be viewed in Appendix E.

The main objective of this phase 1 clinical trial is to test the hypothesis that administration of trans-polydatin with D-lyxohexulose (D-tagatose) does not change the t_{max} or C_{max} of trans-polydatin. All volunteers will be healthy male and female aged 18-30 years old, must not be taking other medications, prescription or nonprescription, with no excessive alcohol intake, and have not previously participated in a clinical trial within the last 30 days. Those potentially eligible subjects meeting the initial inclusion and exclusion requirements, and after signing informed consent, will undergo a basic physical examination, hematology, blood chemistry, and urinalysis. This screening and recruitment process should be complete within 35 days. After meeting all trial criteria, eligible subjects are invited to participate in the trial. They will be asked to attend the trial facility and will be randomly assigned to one of the two treatment groups for the duration of the study. Randomization is to be stratified according to sex to achieve a balanced distribution of subjects across the 2 treatment groups. The treatment groups will be (1) trans-polydatin and (2) BSN272 (trans-polydatin + D-lyxohexulose). Six subjects, 3 male and 3 female, will be assigned to each group for a total of 12 subjects. On the trial day subjects will be given study medication and then timed blood samples will be taken for pharmacokinetic analysis. Urine samples will also be collected during the 12 hours the volunteers are at the CCTS, with one subsequent urine collection at visit 3, which should approximate 24 hours post-treatment, and will be stored for contingent analysis. Serum and urine trans-polydatin and trans-resveratrol will be determined.

For this study, potential subjects will be screened to identify 16 study healthy volunteers (8 male, 8 female) of which 12 participants (6 male, 6 female) will be used for the actual study with 4 alternates (2 male, 2 female) in case a participant cannot complete the study. Six subjects, 3 male and 3 female, will be assigned to each group.

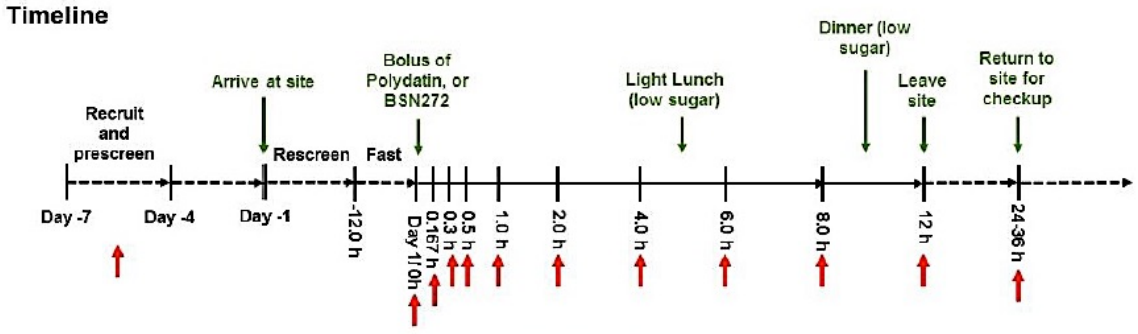
Each subject will drink the study drug(s) assigned to their group dissolved in volume of approximately 4 ounces of water per drug. Each drug will be dissolved separately in water and subjects will drink both solutions (trans-polydatin in the trans-polydatin group, or trans-polydatin and D-lyxohexulose in the BSN272 group) from separate cups in rapid succession. The study dosage is shown in Figure 5.1.

Figure 5.1
Dosing Schedule

Group (Arm)	Treatment	
	Cup #1	Cup #2
Trans-polydatin (n = 6; 3 male, 3 female)	Trans-polydatin (100 µg)	Water
BSN272 (n = 6; 3 male, 3 female)	Trans-polydatin (100 µg)	D-lyxo-hexulose (0.03 g/kg)

After administration of the study drugs, blood samples will then be taken at approximately 0.167 (10 mins), 0.333 (20 mins), 0.5, 1, 2, 4, 6, 8, 12 and once between 24-36 hours post dose. Urine samples will also be collected during the 12 hr clinical period, with one subsequent collection at the 24-hour time point (visit 3). Subjects will be required to attend the trial facility after a 12-hours overnight fast. Subjects will remain in the facility, and monitored constantly for safety for the duration of the timed pharmacokinetic blood collection (12 hours). The subjects will receive a light, low polydatin lunch after the 4-hour blood sample has been taken. After the 12-hour sample draw they will be allowed to leave but will be required to return for the following day for the 24-hour sample collection.

Figure 5.2
Trial Time-line Overview



1. Blood samples (red arrows) drawn prior to and after dosing for quantification of polydatin and D-lyxo-hexulose at recruitment and 24-36 h post-dosing for clinical chemistry and hematology.
2. Urine samples collected for the first12 hours and a final urine sample will be collected post 24 h for contingent analyses.

Determination of Sample Size

Potential subjects will be screened to identify 16 study healthy volunteers (8 male, 8 female) of which 12 participants (6 male, 6 female) will be used for the actual study. There will be 2 treatment groups each having 6 volunteers randomly assigned. The determination of the minimum study group numbers is based upon a 28-day rat toxicology study taken at 1-hour post trans-polydatin, 2-sided, confidence interval of, (alpha 0.025) and a power of 90%.

Statistical Analysis

The primary objective of this phase 1 microdose study is to determine whether the t_{max} and C_{max} of t-PD change significantly when co-administered with DLH in healthy male and female volunteers.

Non-compartmental analysis will be applied to the mean plasma trans-polydatin and trans-resveratrol concentration data from the volunteers. The following parameters will be estimated whenever possible:

1. C_{max} - Maximum observed concentration
2. t_{max} - Time to maximum observed concentration.

C_{max} and t_{max} will be determined by visual inspection of the concentration-time data. If any maxima appear identical, a computer sort will identify the highest data value.

AUC_{0-8} - Area under the concentration-time curve from hour 0 to hour 8, estimated by the linear trapezoidal rule.

AUC_{0-t} - Area under the concentration-time curve from hour 0 to the last measurable concentration, estimated by the linear trapezoidal rule.

$AUC_{0-\infty}$ - Area under the concentration-time curve from hour 0 to infinity for Day 1 calculated as follows:

$$AUC_{0-\infty} = AUC_{0-t} + C_t/\lambda z$$

Where C_t is the last measurable concentration and λz is the elimination rate constant estimated using log-linear regression during the terminal elimination phase. The number of points used in λz calculation will be determined by visual inspection of the data describing the terminal phase. At least the last three time points with measurable values will be used in λz calculation.

M/P ratio Metabolite to Parent ratio calculated as: AUC_{0-8} metabolite/AUC_{0-8} parent pharmacokinetic analysis will be performed using WinNonlin Professional Edition (Pharsight Corporation, Version 5.2). Nominal doses and sampling times will be used. Dose normalized parameters, ratios, and descriptive statistics will be calculated using WinNonlin and Microsoft Excel (Version 11.0). Concentration values found below the lower limit of quantification (< 20.0 ng/mL for trans-polydatin and < 10.0 ng/mL for trans-resveratrol) will be treated as zero for descriptive statistics and toxicokinetic analysis.

A t-test (p=0.05) will be used to calculate the significance of the difference between the C_{max} and t_{max} of t-PD with and without DLH.

The rest of the Phase 1 protocol can be view in Appendix E.

5.5 Conclusions

D-tagatose participated in numerous studies, both clinical and non-clinical, over 2 decades for use as both a low calories food sweetener and subsequently as a treatment for hyperglycemia. These studies led to a GRAS designation for D-tagatose for use in foods and ushered its development as a pharmaceutical, all prior to obtaining an IND designation with the FDA. Our lab was commissioned to navigate the acquisition of the IND for D-tagatose and orchestrate the execution of a Phase III[37] and II[18] trial to access safety and efficacy in the T2DM population. I had the opportunity upon joining the lab off the heels of these trials to parse through the resulting data and contribute their publications into the academic literature.

While the trials showed D-tagatose to be safe and efficacious in lowering blood glucose levels the patent on the molecule came to an end as the FDA requested an additional Phase 3 study as well as a full 2-year cardiotoxicity study due to negative outcomes in some already approved medications for T2DM patients. This made further traditional development of D-tagatose as monotherapy impossible but turned out to be opportunistic, as bringing D-tagatose back into the laboratory and conducting numerous pre-clinical studies combining D-tagatose with 2 potential lipid-lowering candidates proved to be fruitful.

Trans-polydatin and dihydromyricetin were identified through literature searches as potentially good candidates to administer along with D-tagatose (BSN272 and BSN723T respectively) due to studies showing them to have activity in lowering markers of atherosclerotic development including increased blood levels of LDL, total cholesterol, and triglycerides as well as hyperglycemia. This was desirable due to studies showing that increased consumption of carbohydrates leads to greater dyslipidemia than energy matched diets with increased free fatty acids.[43] As cardiovascular disease is the primary cause of mortality in diabetics, this was a concern for the patient population that D-tagatose was intended to treat. In fact, Police *et al*. demonstrated in $LDLr^{-/-}$ mice that D-tagatose administration could produce dyslipidemia when supplemented in feed, although not to the extent as a similar amount of sucrose. Of more concern, 10-months of treatment with D-tagatose in T2DM human patients led to elevations in triglycerides.[37]

A study using doses of D-tagatose and trans-polydatin that were forced to be uncorrelated using principal axis theorem allowed us to determine the contribution of each molecule in the presence of the other molecule towards reductions in markers of dyslipidemia.[45] This allowed for the development of predictive algorithms that aided us in tweaking the doses of each of the molecules to get the maximum affects. Administration of BSN272 led to significant prevention of diet induced elevations in LDL and VLDL as well as weight gain and the development of atherosclerosis. Triglycerides were also lower in the treatment groups throughout the course of the study although it did not reach significance.[45] A follow-up study confirmed the predictive ability of the algorithm, and found similar results, but failed to reach significance again with lowering of triglycerides.[46] In both studies, however, the lowering of triglycerides appeared to be synergistic as neither component of BSN272 as monotherapy showed any activity towards triglycerides.

The combined efficacy of D-tagatose and dihydromyricetin was tested next in a $ApoE^{-/-}$ mouse study. While both molecules appeared somewhat efficacious at lowering total cholesterol and triglycerides as monotherapy, the combination did not appear to be synergistic as BSN723T was not any better than D-tagatose or DMY alone.[49] However, this study did provide a comparator study to assess a longer study with BSN272 to see if this combination product was truly synergistic.

The last push to conduct the lengthy 16 week $ApoE^{-/-}$ mouse study with BSN272 came with results from an unpublished study in Syrian golden hamsters in which monotherapy with polydatin led to marked elevations in triglycerides and the combination of D-tagatose and polydatin led to a significant reduction in triglycerides. The 16-week study was powered specifically for a significant result for prevention of a diet induced increase in triglycerides, and that is what it delivered. Mice given BSN272 showed lower triglycerides at the end of the study then could be expected by combining the results of each as monotherapy, and the results were even below that of the normal diet group.[111] The culmination of these non-obvious findings was in the awarding of a patent for BSN272.

Following these results, we began to prep for an IND submission. Part of this process was conducting a toxicology study for polydatin, as this had already been done for D-tagatose. No

glaring problems arose from these experiments and a NOAEL of 1200 mg/kg/day was established for females and 3000 mg/kg/day for males.[50]

I spent a considerable amount of time after these studies packaging the IND to submit to the FDA and conversing back and forth with them as they added in positioning and submitting our drug candidate. We identified Prader-Willi Syndrome as a potential orphan disease to target as no treatments are available aimed at treating the underlying cause of this disease which begins as inability to obtain satiety and eventually develops into T2DM at a very young age. The future tasks and directions for this work are presented in section 5.6 below.

5.6 Future Work

Much of the work in the near future is pretty straight forward. The submission of an IND is a process that requires communicating with numerous groups of people. The IND package is ready to submit to the FDA once the timing is right for our lab. At that point the FDA has 30 days to respond at which time we can move forward with the Phase 1 study.

Once an IND number is established the protocol will be submitted to the IRB where the study is going to be conducted, most likely by the CCTS at the University of Kentucky. That will require a period of back and forth to get the protocol and other documentation to meet the expectations that they express. The informed consent for volunteers in the study has already been written pending IRB approval, as has the investigators brochure for anyone involved with the study to consult.

All of these documents, including the IND, will require continuous updating and re-submitting to the various regulatory units involved as studies are conducted. A re-submission is required on most documents at least once a year.

Our lab will continue to further pursue Prader-Willi syndrome as an indication. A study is currently underway in our lab of a mouse model of Prader-Willi syndrome using D-tagatose as monotherapy. A pediatric study is also being sought as the safety profile of D-tagatose makes it an interesting candidate for early treatment in individuals with a high likelihood of developing T2DM.

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Appendix A

Safety and Efficacy of D-Tagatose in Glycemic Control in Subjects with Type 2 Diabetes[1]

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Abstract[1]

The primary objectives of this study were to evaluate the treatment effect of D-tagatose on glycemic control, determined by a statistically significant decrease in hemoglobin A1c (HbA1c), and safety profile of D-tagatose compared to placebo. The secondary objectives were to evaluate the treatment effects on fasting blood glucose, insulin, lipid profiles, changes in BMI, and the proportion of subjects achieving HbA1c targets of <7%. Type 2 diabetic patients not taking any blood glucose lowering medications were administered either 15 g of D-tagatose dissolved in 125–250 ml of water three times a day or placebo with meals. Reduction in HbA1c was statistically significant compared to placebo at all post-baseline time points in the ITT population. Additionally, secondary endpoints were achieved in the ITT population with regard to LDL, total cholesterol, fasting blood glucose, and proportion of subjects achieving HbA1c targets of <7%. D-tagatose was unable to lower triglycerides or raise HDL compared to placebo. A subgroup LOCF analysis on the ITT US population showed a greater and statistically significant LS mean reduction in HbA1c in the D-tagatose group at all post-baseline visits. Based on these results it is concluded that in the ITT population D-tagatose is an effective single agent at treating many of the therapy targets of type 2 diabetes including lowering fasting blood glucose and HbA1c, and lowering of LDL and total cholesterol.

Keywords

HbA1c; Triglycerides; Insulin; LDL; HDL; Cholesterol; Blood glucose

Introduction[1]

According to the American Diabetes Association, diagnosed diabetes cost Americans $245 billion in 2012. This includes $176 billion for direct medical costs as well as $69 billion in reduction of productivity.[2] The National Diabetes Statistics Report, 2014, estimates that 29.1 million people, or 9.3% of the United States population, have diabetes (an estimated 8.1 million of these were undiagnosed).[3] From 2009–2012, The Centers for Disease Control and Prevention determined that 37% of U.S. adults aged 20 years or older had prediabetes, as did 51% of adults aged 65 years or older (based on fasting glucose or hemoglobin A1c levels). Extrapolating this percentage to the entire U.S. population during that time, the prevalence of prediabetes can be estimated to be around 86 million American adults.[3]

Two abnormalities that typically characterize the pathogenesis of type 2 diabetes are peripheral insulin resistance and progressive failure of pancreatic β-cell function leading to loss of insulin secretion. These complications eventually lead to chronic hyperglycemia and associated long-term disease complications.[4] Insulin resistance does not progress in parallel with loss of β-cell function, but occurs first. Most type 2 diabetes patients who are initially treated by diet management or oral blood glucose lowering agents eventually require insulin as loss of β-cell function progresses. None of the therapies currently available appear to be able to slow or stop the progression of β-cell loss once it begins. Therefore, there is clearly a need for therapies for the long-term treatment of type 2 diabetes that can prevent patients from reaching this current "point of no return" of loss of β-cell function.

Currently the approach to the treatment of type 2 diabetes is stepwise and systematic with early treatment consisting of having patients alter and manage their diet, adhere to a regular exercise program and, if overweight, control their weight. If the disease progresses and blood glucose remains uncontrolled, then pharmacologic therapy is initiated, usually with one or two oral anti-hyperglycemic drugs. Additional medications or insulin may be added if the disease continues to progress. Many, if not most, patients with type 2 diabetes will eventually require insulin as a primary therapy with or without adjuvant drug therapy in order to control their blood glucose.[5]

Despite the introduction in recent years of new drugs for the treatment of diabetes, glucose control in many patients remains unsatisfactory. Metformin remains the first drug of choice for the treatment of type 2 diabetes. A recent review that included 140 controlled trials and 26 observational studies comparing diabetes medications, both as monotherapy and in two-drug combinations, concluded that the long-term benefits and harms of current drug treatments remain unclear.[6] The study concluded that there was not enough evidence to clearly support the use of one drug or drug combination over another for stemming the complications of diabetes, including macrovascular and microvascular complications, and for mortality. Metformin remains the initial drug of choice, however, because compared to the newer drugs it has the highest benefit-to-risk ratio for intermediate outcomes, such as moderate HbA1c reduction, less weight gain and less risk of hypoglycemia. Many of the other therapies have limiting side effects such as weight gain, hypoglycemia, and edema, and have restrictions for use.[7]

This information illustrates the limitations of drug therapies currently available for the progression of diabetes. Further, even with aggressive intervention, it is estimated that 60% of diabetics do not achieve target blood sugar levels with their current treatment regimen.[5] Moreover, current drugs available for treatment of diabetes may result in unwanted weight gain (long and rapid acting Insulin, sulfonylureas, thiazolidinediones, repaglinide, nateglinide), hypoglycemia (insulin, sulfonylureas), gastrointestinal distress (metformin, α-glucosidase inhibitor, amylin mimetics, bile acid sequestrant, bromocriptine), or more serious adverse events such as pancreatitis (short and long-acting glucagon-like peptide-1 (GLP-1) agonists and dipeptidyl peptidase-4 (DDP-4) inhibitors).[8,9] From the statistics showing a high percentage of diabetics are not able to consistently control their blood sugar levels within recommended limits, it is evident that there is a medical need for a drug that can slow and/or halt the progression of diabetes. Preferably, such a drug should exhibit a unique mode of action to enable additive or synergistic use with current therapies; produce no weight gain, hypoglycemia, or other limiting or unmanageable side effects; preserve or enhance β-cell function; and reduce cardiovascular risk factors that potentially lead to morbidity and mortality.

D-tagatose is an isomer of fructose that is ~90% as sweet as sucrose. In 2001 D-tagatose was designated as a Generally Recognized as Safe (GRAS) product by the United States Food and Drug Administration, and subsequently has been used as a nutritive or low-calorie sweetener.[10] Currently, D-tagatose may be used as a sweetener in diet beverages, light ice creams or yogurts, and regular or dietetic hard candies.[11] Subsequently it was hypothesized from observations in food use, and then demonstrated experimentally that, when consumed, D-tagatose functions as a "sugar blocker" and inhibits lipid formation from carbohydrates without stimulation of pancreatic beta cells for insulin production or secretion.[12] Preliminary animal and pre-clinical studies of D-tagatose demonstrated its ability to lower blood glucose and lipoprotein levels. D-tagatose has been shown to reduce total cholesterol and VLDL and LDL-cholesterol when compared to sucrose,[13] and increase HDL-cholesterol levels.[14] A number of clinical trials demonstrating the ability of D-tagatose to blunt postprandial rises in blood glucose and reduce

HbA1c have been conducted in both healthy subjects and diabetic patients.[14–19] A phase 2 study designed to estimate the lowest dose of D-tagatose capable of lowering HbA1c found that the dose was 5 g TID.[19] Single-dose and repeated-dose studies in healthy and diabetic human subjects have shown that the predominant adverse effects associated with excessive consumption of D-tagatose are gastrointestinal disturbances attributed to osmotic effects from incomplete absorption.[14–18] Such effects are also commonly associated with excessive consumption of other poorly digestible carbohydrates including polyols. In short, D-tagatose provides glycemic and lipoprotein control through a mechanism of action unlike any agent that is currently available on the market in the United States.

Here we report the results of a phase 3 clinical trial with D-tagatose and demonstrate statistically significant reductions in hemoglobin A1c levels (HbA1c) in patients with mild type 2 diabetes. HbA1c levels are an indicator of glycolated hemoglobin in the blood and reflect a patient's glycemic control over a six to twelve week period.[20] If blood glucose levels have been elevated over recent weeks, there will likely be a concomitant rise in HbA1c levels.

The primary objective of this Phase 3 clinical trial was to evaluate the placebo-controlled effect of D-tagatose on glycemic control and safety in subjects with type 2 diabetes over the course of a 10-month treatment. The secondary objectives of this clinical trial were to evaluate the placebo-controlled effects of D-tagatose on fasting blood glucose, insulin, lipid profiles, and changes in BMI. D-tagatose effectively lowered HbA1c levels in type 2 diabetic patients compared to placebo; thus, results of this phase 3 clinical trial illustrate the potential for D-tagatose to fulfill a need in current diabetes treatment.

Patients and Methods[1]

Ethical Conduct of the Study

The protocol and protocol amendment(s) were reviewed and approved by an Institutional Review Board (IRB) before the study was initiated. This trial was conducted in accordance with regulations governing clinical trials including the US Code of Federal Regulations (CFR), Title 21, Part 50; regulations governing IRBs, Title 21, Part 56; and the Declaration of Helsinki concerning medical research in humans (Recommendations Guiding Physicians in Biomedical Research Involving Human Patients: adopted by the 18th World Medical Assembly (WMA), Helsinki, Finland, June 1964 and amended by the 29th WMA, Tokyo, Japan, October 1975, the 35th WMA, Venice, Italy, October 1983, the 41st WMA, Hong Kong, September 1989, the 48th WMA, Somerset West, Republic of South Africa, October 1996, and the 52nd WMA, Edinburgh, Scotland, October 2000). Additional governing regulations included US CFR Title 21, Part 54 and US CFR Title 21, Part 312. This study was also conducted according to International Conference on Harmonization (ICH) Good Clinical Practices (GCP).

Eligibility

Subjects were required to meet the following criteria for inclusion in this study: male or female between the ages of 18 and 75 diagnosed with type 2 diabetes (according to WHO criteria) who were being treated with diet and exercise alone, and not on any medication for diabetes; a HbA1c level at screening and baseline greater than 6.6% and less than 9.0%; a fasting glucose concentration less than 240 mg/dL (13.3 mmol/l); a BMI of less than or equal to 45 kg/m2; and a stable weight (±10%) for 3 months prior to entry into the study. Exclusion criteria included: treatment with any sulfonylureas, or other antidiabetic medications (e.g., thiazolidinediones, metformin, acarbose, exenatide, or insulin) within the prior 3 months; chronic (lasting longer than 14 consecutive days) systemic glucocorticoid treatment within 4 weeks of the baseline visit; use

of any weight loss drugs within the prior 3 months; proliferative retinopathy; known or suspected abuse of alcohol or narcotics; any experience with hypoglycemic unconsciousness; impaired hepatic, renal or cardiac function; uncontrolled hypertension; pregnancy, breastfeeding, or intention of becoming pregnant or judged to be using inadequate contraception; documented gastrointestinal disease, or taking of medications to alter gut motility or absorption; and treatment with any investigational drug within 30 days of the screening visit.

Three populations were evaluated:

- The Intent-to-Treat (ITT) population consisted of all randomized subjects who received at least one dose of their randomized treatment and had at least one post-treatment visit evaluating efficacy.
- The Per Protocol (PP) population consisted of all ITT subjects who had at least 80% compliance with medication for 75% of the dosing time points and had no major protocol violations. Subjects who had a screening and visit 2 HbA1c value ≤ 6.6% and ≥ 9.0% and who were put on other diabetes medications were excluded from PP population.
- The Safety population consisted of all randomized subjects who received at least one dose of their randomized treatment and had at least one post-treatment visit evaluating safety.

Treatment protocol

This was a Phase 3, multicenter, randomized, double-blind, placebo-controlled, parallel-group study to evaluate the efficacy, safety, and tolerability of D-tagatose.

Subjects were screened for eligibility using physical examination and clinical laboratory tests. The basic physical examination included physical measurements, general examination by observation (inspection), palpation, percussion, auscultation, blood pressure measure, and heart rate check. The clinical laboratory tests included: (1) hematology (hematocrit, hemoglobin, MCH, MCHC, MCV, total white blood cells, platelets, and differential); (2) clinical chemistry (sodium, chloride, potassium, carbon dioxide, BUN, uric acid, albumin, creatinine clearance, SGOT, SGPT, bilirubin (total and direct), phosphorus, calcium, alkaline phosphatase, total protein, and glucose (fasting); (3) HbA1c; (4) serum lipid profile including total cholesterol, HDL, LDL, and triglycerides; (5) urinalysis (appearance, volume, specific gravity, pH, glucose, protein, and microscopic evaluation of urinary sediment). Those determined to be provisionally eligible participated in an 8-week lead-in period prior to the start of the study during which diabetes education was provided and diet and exercise treatment stabilized.

Patients recorded their food intake and exercise in nutrition diaries. After the 8 week lead-in period, fasting (minimum of 8 hours) subjects returned to the study sites and underwent medical history review followed by baseline tests including ECG (or EKG), pregnancy test for females, and hematology, clinical chemistry, and urinalysis tests. The clinical laboratory tests were the same as those performed during the screening visit. Subjects continued on a weight-maintaining diet plus exercise under physician's recommendation. In addition, subjects received their randomized study treatment and detailed instructions about its use. A total of 494 subjects were randomized into the study. Randomization was stratified according to screening HbA1c values (<7.5% and ≥7.5%) to achieve a balanced distribution of subjects across two arms (treatment and placebo).

The treatment period consisted of 12 monthly visits, the first (Visit 2, designated month 0 in figures) of which was used to gather the baseline data for the efficacy and safety parameters and also included the first distribution of test and placebo treatments. Baseline was defined as the

last available value before the first randomized treatment. Subsequent visits occurred monthly and were of two types: (1) Supply Visits and (2) Supply and Procedures Visits. HbA1c was monitored at baseline (Visit 2) and every 2 months thereafter as were the secondary end-point parameters (described below). Efficacy analyses were conducted on data from 2, 6, and 10 months.

There were two treatment groups in this trial: drug (D-tagatose), and placebo (Splenda). The dose of D-tagatose was 15 g dissolved in 125 to 250 mL of water TID; the dose of placebo was 1.5 g dissolved in 125 to 250 mL of water TID. The placebo amounts were chosen to match sweetness for blinding. The powder packets were the same size and bore the same labeling with the exception of the designation "Substance A" or Substance B". The entire drug/placebo solution was consumed prior to each main meal (breakfast, lunch, dinner). If severe gastrointestinal (GI) effects were seen, the tagatose dosage was reduced to 10 g TID temporarily and further reduced to 5 g TID if severe GI side effects were not resolved within 24 hours. The dosage of D-tagatose would then be increased to 10 g TID and further increased to 15 g TID when patients had adapted to the treatment *(i.e.,* GI effects reduced to mild). The placebo dosage would be reduced in a similar fashion if severe GI effects were seen. That is, the dose could be reduced to 1.0 g TID temporarily and then further reduced to 0.5 g TID if severe GI side effects were not resolved within 24 hours. The dosage of placebo was then to be increased to 1.0 g TID and further increased to 1.5 g TID when patients had adapted to the treatment (i.e., GI effects reduced to mild).

Removal of Subjects from Therapy or Assessment

Premature end-point: A premature end-point of the efficacy analysis for the trial occurred when additional antidiabetic medications were prescribed to a patient at the sole discretion of their primary care physician. However, patients were advised to continue in the trial if an antidiabetic medication was added after the start of the trial, even though the HbA1c data were not used in efficacy analyses at the time points subsequent to the initiation of the additional medication(s). The continuation of the trial was solely for the safety analysis of D-tagatose and patient's data were withdrawn from the efficacy analysis.

Treatment failures: Subjects were categorized as treatment failures if both of the following criteria were met:

1. HbA1c change of +1.0% from baseline at any clinic visit or an HbA1c ≥10% or required additional antidiabetic medication for glycemic control;

2. Received at least 80% of all study drug doses within the initial 3 month dosing period.

The need for additional antidiabetic medication was determined by an elevated fasting blood glucose value (>240 mg/dL) not secondary to a readily identified illness or pharmacological treatment. Investigators were to withdraw subjects from study treatment (and therefore the evaluable population for assessment of efficacy as measured by HbA1c) after additional antidiabetic medication had been prescribed. However, subjects were advised to continue the rest of the trial procedures for the assessment of safety parameters.

Criteria for Evaluation

Efficacy: The primary efficacy variable was the change from baseline in HbA1c level when measured at pre-specified time points. Efficacy analyses were conducted on data from 2, 6 and 10 months (visit 4, 8 and 12). Statistical analyses were conducted on the 2-month, 6-month and 10-month data separately. The primary efficacy endpoint was at 2 months in the Intent-To- Treat population. Secondary efficacy endpoints for change in HbA1c were at 6 and 10 months.

Additional secondary efficacy end-points were measured at the same time points and included changes from baseline in (1) fasting blood glucose, (2) serum insulin levels, (3) lipid profiles (LDL, triglycerides, total cholesterol, HDL), and (4) BMI, as well as the proportion of subjects achieving HbA1c targets of <7%.

Safety: The safety parameters assessed for each subject included adverse events, serious adverse events, vital signs, results of physical exams, 12-lead ECG, and clinical laboratory tests (hematology, chemistry, and urinalysis).

Statistical Methods: Three analysis populations were evaluated: (1) The Intent-to-Treat (ITT) population, (2) the Per Protocol (PP) population, and (3) the Safety population. The ITT population was the focus of the efficacy analyses for regulatory purposes, with the PP population being used in supportive analyses. For the ITT population, missing data (including missing values at intermediate visits) were to be imputed from scheduled visits using the last-observation-carried-forward (LOCF) method.

The Safety population was used for analyses of all safety parameters. Secondary efficacy end-points were evaluated using the closed test procedure (in order to preserve the type I error rate, α, of 0.05). Accordingly, the analysis of end-points was conducted in the following order: (1) body mass index, (2) triglycerides, (3) LDL, (4) total cholesterol, (5) fasting blood glucose, (6) proportion of subjects with HbA1c concentration of <7%, (7) insulin, and (8) HDL. Analysis of continuous efficacy variables used mixed-model repeated-measures analysis of covariance controlling for covariates and stratification factors identified as significant in preliminary explorations. Categorical efficacy variables were analyzed using general estimating equation models.

Results[1]

Study Population

There were 494 subjects randomized, 185 subjects in the US and 309 subjects in India. Of these, 480 were treated, 248 with placebo and 232 with D-tagatose (Table 1). The mean age of subjects in the ITT population was 52 with an age range between 22 and 74. The ITT population was approximately evenly divided between males and females and the racial distribution was 72% Asian, 12% Caucasian, 11% Latino and 5% Black, with approximately equivalent distributions in the D-tagatose and placebo groups. At baseline, approximately 90% of subjects in both treatment groups were controlling their diet and exercising in order to control their diabetes.

Table 1[1]

Analysis Population

Population	Placebo N = 253 n (%)	D-tagatose N = 241 n (%)	Total N = 494 n (%)
ITT	184 (72.7%)	172 (71.4%)	356 (72.1%)
Per Protocol	119 (47.0%)	85 (35.3%)	204 (41.3%)
Safety	207 (81.8%)	185 (76.8%)	392 (79.4%)

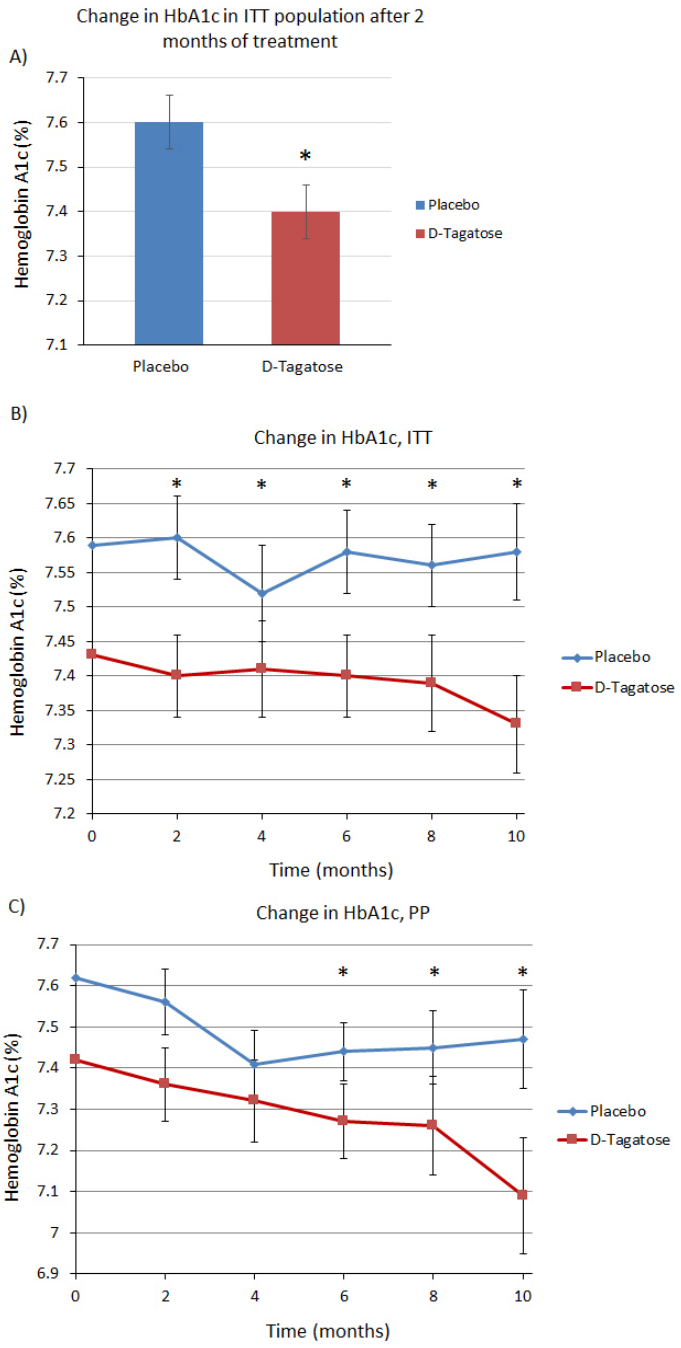
Efficacy Results

Primary Endpoint Results: Decrease in Hemoglobin A1c Level: The primary efficacy variable was the change from baseline in HbA1c level when measured at pre-specified time points. An efficacy analysis was conducted on data from 2, 6 and 10 months (Visits 4, 8 and 12). The primary efficacy

end-point, set at the 2-month time point in the ITT population, showed a statistically significant difference ($p = 0.0198$) between D-tagatose and the placebo (Figure 1A). In the ITT population, the active treatment group, i.e., subjects who received D- tagatose, showed greater and statistically significant reductions in HbA1c levels at all post-baseline visits when compared to the placebo group (Figure 1B). The same mixed model analysis conducted with the PP population data showed similar results insofar as the population receiving D-tagatose always showed greater decreases in HbA1c compared to placebo (Figure 1C). The differences in the PP population did not achieve statistical significance until month 6, however, but then remained significant throughout the remainder of the study.

Analysis of subgroups: In light of the above results, subgroup analyses were conducted to examine whether starting HbA1c levels (< 7.5% or ≥ 7.5%) influenced the effectiveness of D-tagatose. The results of these analyses for the ITT population (LOCF) are shown in Figure 2A and 2B. A significant difference between D-tagatose- and placebo-treated subjects was seen earlier in the subgroup entering with a baseline HbA1c < 7.5%., although both subgroups achieved greater changes from baseline than those observed in the placebo groups.

Figure 1[1]

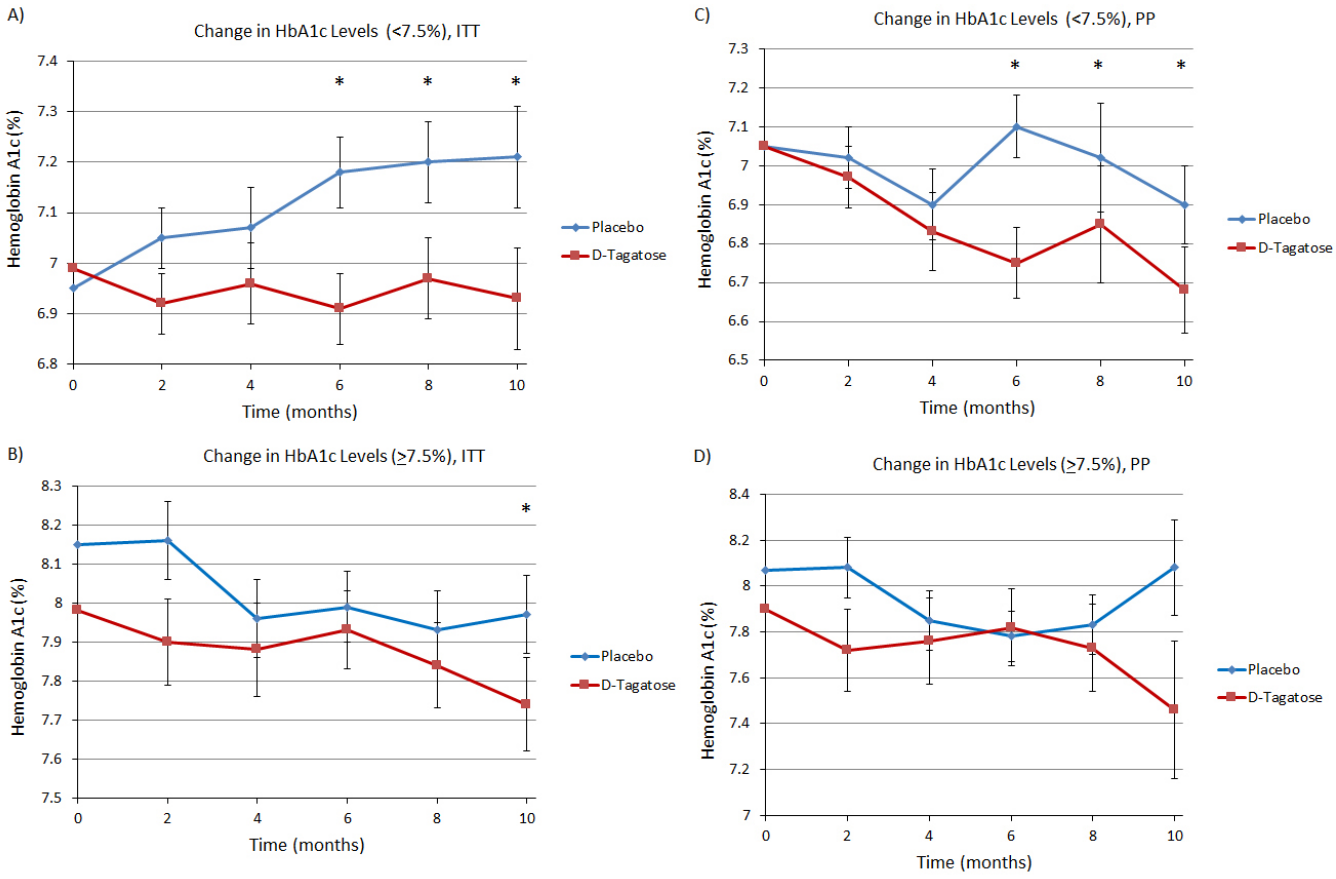


(A) Change in HbA1c in ITT population after 2 months of treatment. The primary efficacy endpoint set at 2 months in the ITT population showed a significant difference between placebo and D-tagatose groups (p=0.0198) (placebo n=182, D-tagatose n=172). (B) Change in HbA1c in ITT population. Significant differences were seen at 2 (p=0.0198), 4 (p=0.0160), 6 (p=0.0015), 8 (p=0.0002), and 10 months (p=<0.0001). (C) Change in HbA1c in PP population. Significant differences were seen at 6 (p=0.0343), 8 (p=0.0148), and 10 months (p=0.0021). Zero time points plotted as means, remaining time points plotted as least squares means ±SEM.[1]

In the PP population the D-tagatose treatment for both subgroups demonstrated greater reductions in HbA1c compared to placebo (with the exception at 6 months in the ≥ 7.5% subgroup) (Figure 2C and 2D). However, a statistically significant difference between the D-tagatose and

placebo-treatment groups was only achieved in the <7.5% subgroup (Figure 2C). Statistical significance between D-tagatose and placebo groups was achieved after 6 months of treatment in subjects with a baseline HbA1c of <7.5% (Figure 2C, p-values of 0.0497, 0.029, and 0.0121 after 6, 8, and 10 months of treatment, respectively), whereas statistical significance between D-tagatose and placebo groups was not achieved in patients with a higher baseline HbA1c at any time point (Figure 2D).

Figure 2[1]

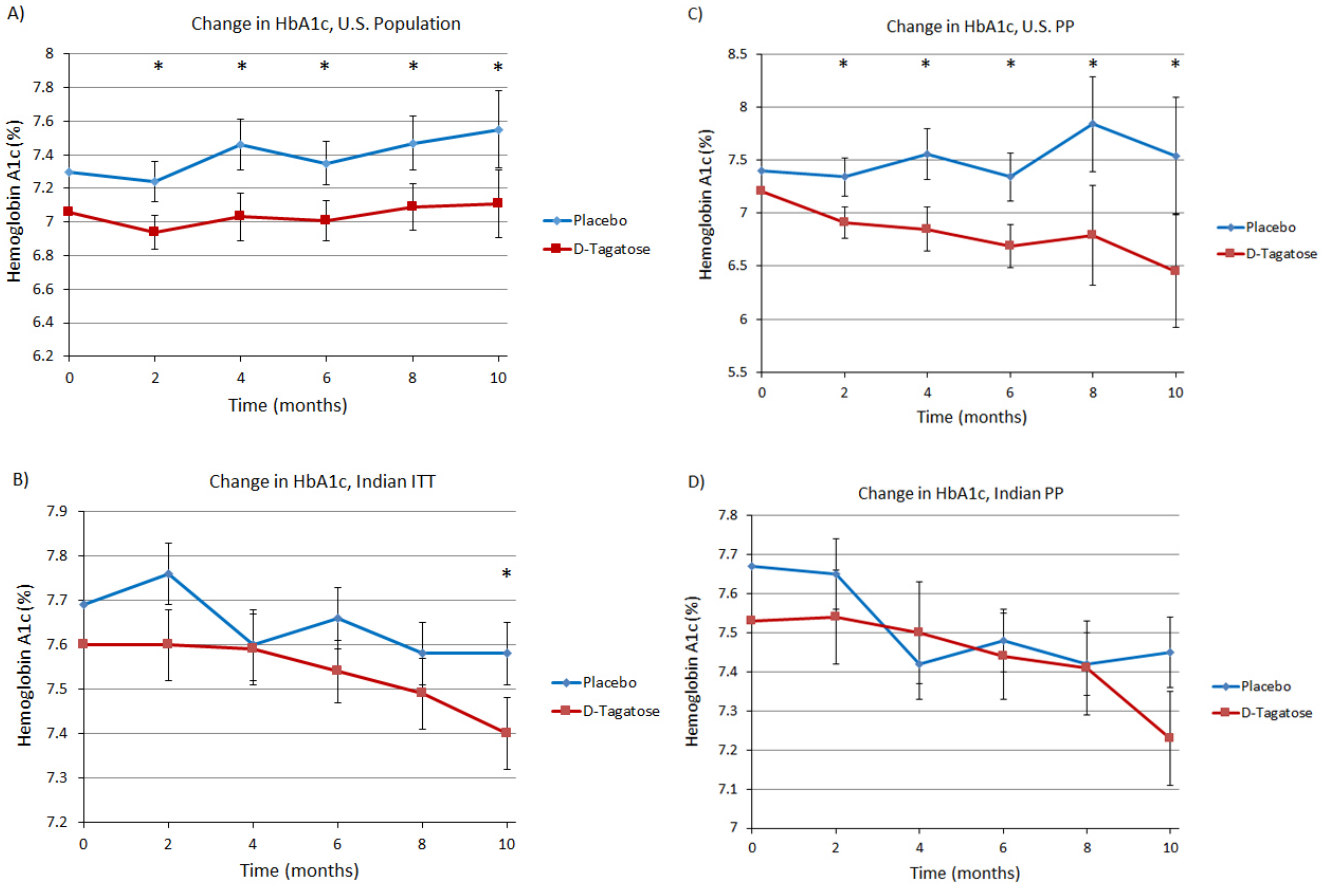


(A) Results of analyses for the ITT population with starting HbA1c baseline < 7.5%. Significant differences between D-tagatose and placebo were seen at 6 (p=0.0030), 8 (p=0.0004), and 10 months (p=<0.0001). (B) Change in HbA1c in ITT subpopulation with baseline ≥7.5%. A significant difference between D-tagatose and placebo was seen at 10 months (p=0.0277). (C) Change in HbA1c in PP subpopulation with baseline HbA1c <7.5%, (D) Change in HbA1c in PP subpopulation with baseline ≥7.5%. Zero time points plotted as means, remaining time points plotted as least squares means ±SEM.[1]

Further subgroup analyses were conducted to compare the ability of D-tagatose to effectively decrease HbA1c in the United States (US) population of subjects versus subjects of Indian origin. Interestingly, the effect on lowering HbA1c was more pronounced in US compared to Indian subjects (Figure 3). A subgroup analysis on the ITT US population (Figure 3A) showed a greater and statistically significant LS mean reduction in HbA1c in the D-tagatose groups than in the placebo group at all post-baseline visits: month 2 (reduction of 0.23 vs. an increase of 0.07, Δ = 0.3 and p = 0.0273), month 4 (reduction of 0.15 vs. an increase of 0.28, Δ = 0.4 and p = 0.0011), month 6 (reduction of 0.17 vs. an increase of 0.17, Δ = 0.3 and p = <0.0001), month 8 (reduction of 0.10 vs. an increase of 0.29, Δ = 0.4 and p = <0.0001), and month 10 (reduction of 0.07 vs. an increase of 0.37, Δ = 0.4 and p = <0.0001). Analysis of the LOCF ITT data showed statistically

significant reductions in the India population only at month 10 (Δ = 0.2, p = 0.0187) (Figure 3B). PP analyses for the US population produced similar results with statistically significant differences being noted at all post-baseline time points (Figure 3C). The effect of D-tagatose in reducing HbA1c was evident by 2 months of treatment in the US subjects. However, the effect of D-tagatose in reducing HbA1c in the Indian subjects was not significantly different than placebo at any time point in the PP population (Figure 3D).

Figure 3[1]



(A) Change in HbA1c U.S. subjects, ITT population. (B) Change in HbA1c, Indian subjects, ITT population. (C) Change in HbA1c U.S. subjects, PP population. p-values for 2, 4, 6, 8, and 10 months of treatment are 0.0435, 0.0016, <0.0001, <0.0001, and <0.0001, respectively. (D) Change in HbA1c, Indian subjects, PP population. Zero time points plotted as means, remaining time points plotted as least squares means ±SEM.[1]

Proportion of Subjects Achieving HbA1c < 7%: The data from the ITT population with LOCF indicated that the D-tagatose group had a greater proportion of subjects achieving an HbA1c level of less than 7% at all post baseline time points compared to placebo, with the results being statistically significant at post-baseline months 6, 8 and 10 (Table 2). Thus, the percent of responders in the D-tagatose group was higher than in the placebo group at 6 months (38.37% vs 22.4%), 8 months (40.7% vs 25.68%) and 10 months (43.02% vs 26.23%, respectively). Similar results were observed in the analyses using the PP population except that in the latter case statistical significance was observed at months 6 and 10 (Table 3).

Table 2[1]

Proportion of Subjects Achieving HbA1c <7%, ITT Population

Month	Statistic	Placebo	D-tagatose	Delta	p-values
2	Responder	49 (26.92%)	65 (38.01%)	11.09	0.0913
	Non-responder	133 (73.08%)	106 (61.99%)		
4	Responder	53 (28.96%)	68 (39.53%)	10.57	0.1580
	Non-responder	130 (71.04%)	104 (60.47%)		
6	Responder	41 (22.40%)	66 (38.37%)	15.97	0.0048
	Non-responder	142 (77.60%)	106 (61.63%)		
8	Responder	47 (25.68%)	70 (40.70%)	15.01	0.0133
	Non-responder	136 (74.32%)	102 (59.30%)		
10	Responder	48 (26.23%)	74 (43.02%)	16.79	0.0052
	Non-responder	135 (73.77%)	98 (56.98%)		

Table 3[1]

Proportion of Subjects Achieving HbA1c <7%, PP Population

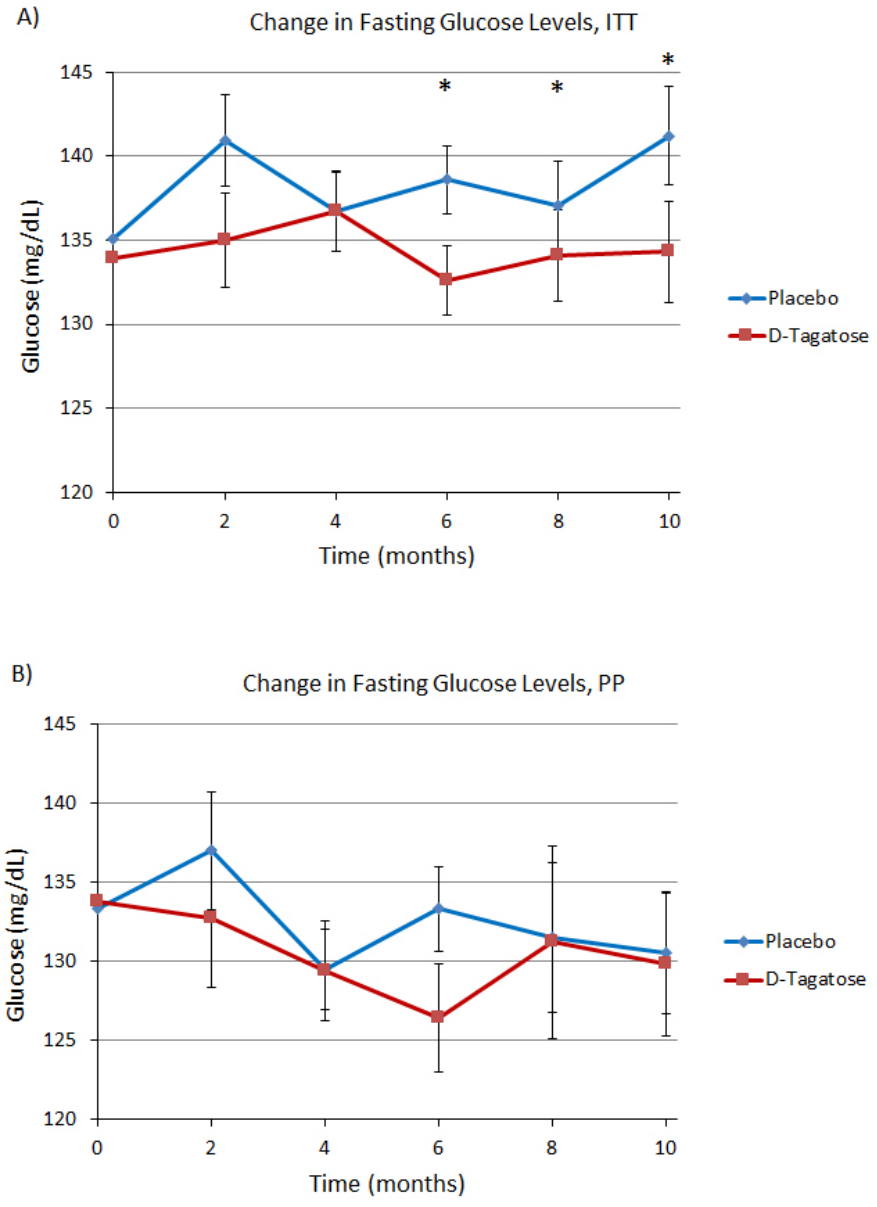
Month	Statistic	Placebo	D-tagatose	Delta	p-values
2	Responder	31 (26.50%)	32 (38.10%)	11.60	0.3787
	Non-responder	86 (73.50%)	52 (61.90%)		
4	Responder	31 (30.39%)	29 (43.94%)	13.55	0.5260
	Non-responder	71 (69.61%)	37 (56.06%)		
6	Responder	19 (21.11%)	27 (48.21%)	27.10	0.0073
	Non-responder	71 (78.89%)	29 (51.79%)		
8	Responder	21 (28.00%)	21 (46.67%)	18.67	0.1052
	Non-responder	54 (72.00%)	24 (53.33%)		
10	Responder	14 (25.93%)	22 (57.89%)	31.97	0.0044
	Non-responder	40 (74.07%)	16 (42.11%)		

Secondary Endpoint Results: Changes in BMI, fasting blood glucose, insulin, blood lipids: D-tagatose was also evaluated for its placebo-controlled treatment effects on BMI, fasting blood glucose, insulin, blood cholesterol, and triglyceride levels. There was no observed effect of D-tagatose treatment on changes in body weight or BMI (body mass index) compared to placebo in either the ITT or PP populations.

For the ITT population better reductions in fasting blood glucose levels from baseline were observed in the D-tagatose group compared to the placebo group at all post baseline time points (Figure 4A), with the differences in LS mean being statistically significant for post-baseline month 6 and beyond, i.e., month 6 (reduction of 2.0 vs. an increase of 4.1, $\Delta = 6.0$ and $p = 0.0440$), month 8 (reduction of 0.44 vs. an increase of 2.5, Δ 2.9 and $p = 0.0340$), and month 10 (reduction of 0.25

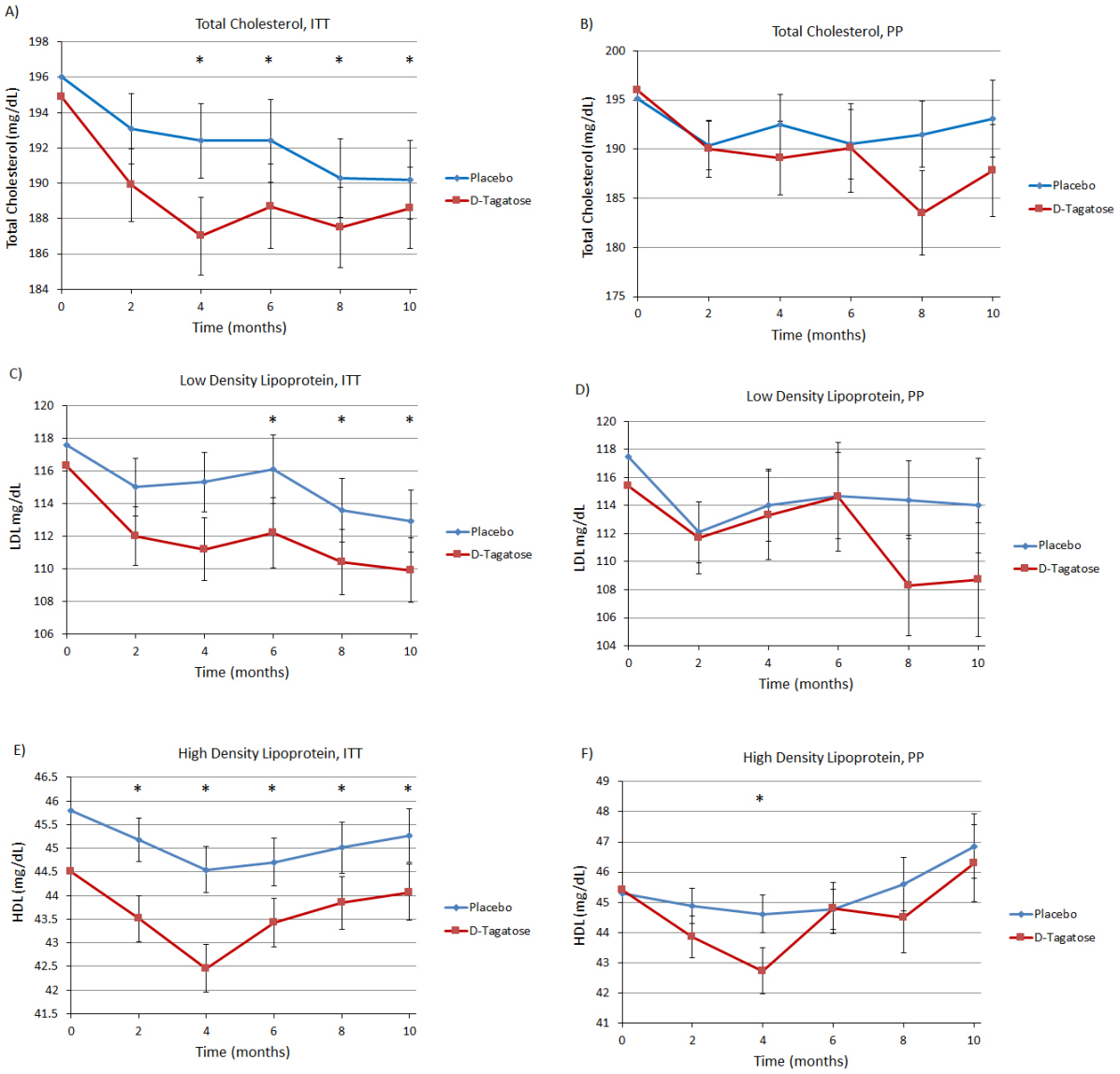
vs. an increase of 6.6, Δ = 6.9 and p = 0.0079). Reductions were also seen in PP population analyses; however, none were statistically significant (Figure 4B). Regardless of the statistically significant difference in fasting blood glucose between placebo and D-tagatose groups, there was no detectable consistent change in serum insulin concentrations in this clinical trial (data not shown).

Figure 4[1]



(A) Change in fasting blood glucose, ITT population. D-tagatose significantly decreased fasting blood glucose compared to placebo after 6 months of treatment. (B) Change in fasting blood glucose levels, PP population. No significant difference between placebo and D-tagatose groups was observed at any time point. Zero time points plotted as means, remaining time points plotted as least squares means ±SEM.[1]

Figure 5[1]



(A) Total cholesterol, ITT population. (B) Total cholesterol, PP population. (C) LDL, ITT population. (D) LDL, PP population. (E) HDL, ITT population. Significant differences between D-tagatose and placebo were seen at 2 (p=0.0126), 4 (p=0.00040, 6 (p=0.0002), 8 (p=0.0003), and 10 months (p=0.0008). (F) HDL, PP population. The only significant difference was at 4 months (p=0.0397). Zero time points plotted as means, remaining time points plotted as least squares means ±SEM.[1]

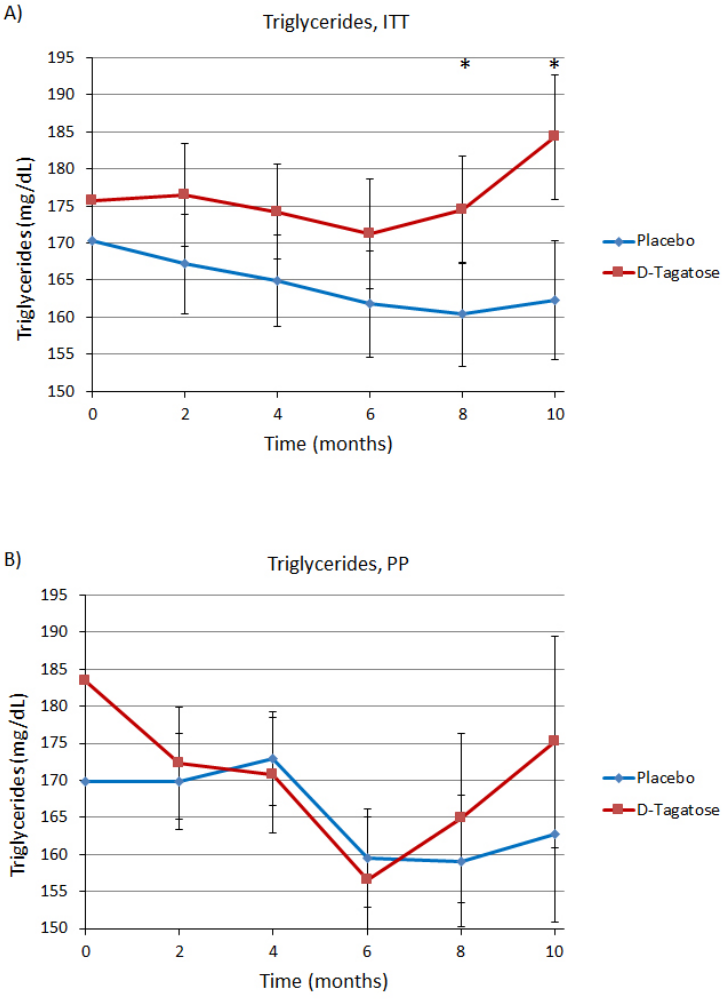
Particularly interesting findings of this phase 3 clinical trial include the effect of D-tagatose to significantly reduce total cholesterol and LDL-cholesterol compared to placebo. The D-tagatose group (ITT population) showed better reductions in total cholesterol from baseline compared to placebo at all post baseline time points (Figure 5A), with the differences becoming statistically significant after 4 months of treatment. This effect was maintained for the duration of the trial (differences in LS means being statistically significant starting from month 4 (reductions of 8.5 vs. 3.2, Δ = 5.3 and p = 0.0402), month 6 (reductions of 6.9 vs. 3.2, Δ = 3.7 and p = 0.0217), month 8 (reductions of 8.1 vs. 5.3, Δ = 2.8 and p = 0.0157), and month 10 (reductions of 6.9 vs. 5.4, Δ = 1.5

and p = 0.0176). In the PP population analyses, reductions were also seen; however, none were statistically significant (Figure 5B).

Better reductions from baseline in LDL levels were observed in the D-tagatose-treated group (ITT population) than the placebo at post baseline time points (Figure 5C), with the differences in LS mean being at month 6 a reduction of 4.8 vs. 0.96, Δ = 3.8 and p = 0.0211; at month 8 a reduction of 6.6 vs. 3.6, Δ = 3.0 and p = 0.0100; and at month 10 a reduction of 7.1 vs. 4.2, Δ = 2.9 and p = 0.0057. Smaller reductions were seen in the PP population analyses and none were statistically significant (Figure 5D).

The results of analyses of serum HDL concentrations in the ITT population showed greater decreases in the D-tagatose group than in the placebo group with the differences being statistically significant at all post-treatment time points in the ITT population and at the month 4 time point in the PP population (Figure 5E and 5F). However, the mean HDL values always remained above the target value of 40 mg/dL so it is unclear whether the reduction in HDL concentrations observed in the D-tagatose group has any clinical significance.

Figure 6[1]



Serum triglycerides. (A) ITT population. (B) PP population. Zero time points plotted as means, remaining time points plotted as least squares means ±SEM.[1]

Triglyceride levels showed no benefit from D-tagatose treatment, *i.e.*, the placebo group showed greater reductions in triglyceride levels than the D-tagatose group with the differences becoming statistically significant from month 8 onward (i.e., at month 8 a reduction of 12.7 vs. an increase of 1.46, $\Delta = 14.2$ and $p = 0.0301$; and at month 10 a reduction of 10.8 vs. an increase of 11.2, $\Delta = 22$ and $p = 0.0048$ (Figure 6A)). No statistically significant differences were seen in the PP population analyses (Figure 6B). Additionally, subgroup analyses for subjects who had baseline triglyceride levels 1) less than 200 mg/dL, 2) between 200 and 500 mg/dL, and 3) above 500 mg/dL, revealed no statistically significant differences between the D-tagatose and placebo treatment groups (data not shown).

Safety Results: D-tagatose was reasonably well tolerated by the subjects in this trial with most of the adverse events (AEs) experienced being mild to moderate in intensity. Adverse events in the D-tagatose treatment group were similar to that of the placebo group, with most instances noted as GI disturbances. Importantly, there were no reported episodes of hypoglycemia or pancreatitis.

The incidence of SAEs in the D-tagatose group was about one-half that seen in the placebo group. The incidences of AEs by system organ class were generally similar between the placebo and D-tagatose groups, the one exception being AEs in the gastrointestinal disorders class. Most of the subjects in both treatments experienced GI symptoms; however, the incidence was higher in the D-tagatose group, and the GI AEs were more frequently severe in the D-tagatose group. There were no remarkable effects in other safety parameters with very few clinically significant changes in safety laboratory values, ECG parameters, vital signs or physical examinations.

Discussion

This trial met its primary objective of demonstrating that D-tagatose was effective at reducing the HbA1c level when administered for two months at doses of 15 g TID. The secondary end-point of significant reductions in the HbA1c level at six and ten months were also met. Mixed model analyses using the ITT population and LOCF imputation of missing values indicated significantly greater reductions in the mean HbA1c levels in the D-tagatose group than in the placebo group at all post-baseline visits. The same mixed model analysis conducted with the PP population data showed similar results insofar as the population receiving D-tagatose always showed greater decreases in HbA1c compared to placebo. Additionally, subgroup analyses were done for the primary end-point on different subgroups of subjects to see if the reduction in HbA1c is significantly different between the treatment groups in the different subgroups *i.e.*, 1) subjects whose baseline HbA1c level was less than 7.5%, 2) subjects whose baseline HbA1c level was greater than or equal to 7.5%, 3) subjects who were randomized in India, and 4) subjects who were randomized in US. In these analyses the results were generally agreement with the primary results although in the India subgroup the results were statistically significant at far fewer time points. Additionally, it was observed that a greater proportion of subjects in the D-tagatose group had achieved HbA1c targets of <7%; this difference was also found to be statistically significant for the majority of the post-baseline time points. Regardless of the subgroup analyzed, the effect of D-tagatose to decrease HbA1c levels was robust and could be seen with differing analytical approaches. These results demonstrate the ability of D-tagatose to effectively aid in the control of blood sugar levels for type 2 diabetics and suggest D-tagatose as a potential drug therapy in this area.

Statistically significant differences were observed between the D-tagatose and placebo treatment groups for the secondary end-points LDL, total cholesterol, and fasting blood glucose when examining the ITT population. Reductions were also seen in PP population analyses, however

none were statistically significant. Numerous studies have demonstrated that increased total cholesterol and particularly elevated LDL-cholesterol are associated with amplified risk for the development of cardiovascular disease. Thus, this finding is especially important for type 2 diabetic patients that may also battle dyslipidemia and/or the metabolic syndrome.[21]

There were also two end-points, triglycerides and HDL levels, where the D-tagatose treatment produced greater and statistically significant increases in the case of triglycerides and decreases in the case of HDL levels compared to the placebo group in the ITT population. The PP population however, showed no significant difference in triglycerides or HDL between placebo and D-tagatose. Of note, a recent study by Donner *et al*. demonstrated a striking and significant effect of D-tagatose to increase HDL from a baseline level of 30.5 ± 15.8 to 41.7 ± 12.1 mg/dL ($p<0.001$) after 10 months of treatment in 6 subjects who did not take lipid medications during the study.[14] Beginning HDL levels in the current study were higher (mean placebo 45.8, D-tagatose 44.5 in ITT and mean placebo 45.5, D-tagatose 45.5 in PP) than in the Donner study. The differences in results between these studies could be from this initial HDL level discrepancy, and any elevations in HDL that D-tagatose is able to provide may only manifest if HDL levels are below recommended values. Consequently, further studies are needed to elucidate the direct effects of D-tagatose on HDL cholesterol levels. HDL-cholesterol levels are inversely related to risk of developing cardiovascular disease.

There was no observed effect of D-tagatose treatment on changes in body weight or BMI (body mass index) compared to placebo in either the ITT or PP populations. In contrast to these findings, previous studies in humans[14] and rodents[13] have demonstrated significant weight loss and a reduction in body weight gain with D-tagatose, respectively. The mean body weight of subjects (both D-tagatose and placebo groups) included in this phase 3 clinical trial at baseline was 73.8 kg, or 162 pounds; with an average BMI of 28.3 mg/m2 (overweight category). However, in previous studies which have demonstrated an effect of D-tagatose to promote weight loss, the mean body weight of all subjects at baseline was much higher (mean body weight at baseline ~109 kg, or 240 pounds.[14] Thus, it is plausible that the subject population within this phase 3 clinical trial was not adequately overweight to demonstrate an effect of D-tagatose to promote weight loss.

D-tagatose appeared to be reasonably well tolerated by the subjects in this trial with most of the AEs experienced being mild to moderate in intensity. The incidence of SAEs in the D-tagatose group was about one-half that seen in the placebo group. The incidences of AEs by system organ class were generally similar between the placebo and D-tagatose group, the one exception being AEs in the gastrointestinal disorders class. Most of the subjects in both treatments experienced GI symptoms; however, the incidence was generally higher in the D-tagatose group, and the GI AEs were more frequently severe in the D-tagatose group. There were no remarkable effects in other safety parameters with very few clinically significant changes in safety laboratory values, ECG parameters, vital signs or physical examinations. Previous single-dose and repeated-dose studies in healthy and diabetic human subjects showed that the predominant adverse effects associated with consumption of high levels D-tagatose were gastrointestinal disturbances attributed to osmotic effects from incompletely absorbed tagatose.[14–19,22–24] At single doses of up to 25 g D-tagatose per meal, flatulence was generally the only side effect, with nausea, borborygmi (i.e., rumbling or gurgling noises, colic, and laxation noted at higher doses). Such effects are also commonly associated with excessive consumption of other poorly digestible carbohydrates.

In summary, results of this phase 3 clinical trial demonstrate an effect of D-tagatose to significantly decrease HbA1c levels over time compared to placebo. The longer subjects remained on the treatment, the further HbA1c was reduced. D-tagatose treatment also reduced fasting blood glucose levels and total and LDL-cholesterol concentrations.

Conclusions

- D-tagatose was effective at lowering HbA1c levels when administered at a daily dose of 15 g in 125- 250 mL of water three times per day just prior to meals.
- Unlike many other diabetes drugs, the longer a patient is on D-tagatose therapy in compliance with instructions, the better the efficacy.
- The effect on HbA1c levels was robust and could be demonstrated in both subgroups, various subpopulations, and with differing analytical approaches.
- D-tagatose appeared to be reasonably well tolerated with most of the AEs experienced in both treatment groups being mild or moderate in severity.

Bloomgarden et al.'s analysis of 61 studies shows that the degree of decrease in HbA1c produced by drugs for treating type 2 diabetes is dependent on the baseline HbA1c; the higher the baseline the greater the decrease. When the baseline HbA1c was <8.0%, the reduction from active therapy is only 0.1–0.2% greater than in the control group. When the HbA1c was >8.0 to 11.8 the reduction in HbA1c was between -0.6 and -1.2%, and the greater the HbA1c, the greater the reduction overall.[25]

In this study the mean HbA1c baselines were 7.6% ± 0.75 (± stdev) and 7.4% ± 0.59 for placebo and D-tagatose groups respectively in the ITT population, and 7.7% ± 0.64 and 7.5% ± 0.53 for placebo and D-tagatose groups respectively in the PP population. Based on Bloomgarden's study (see Table 1 in Bloomgarden)[25], other drugs show an average of only half the efficacy of D-tagatose in similar patients. Overall results of this phase 3 clinical trial suggest a strong potential for D-tagatose as an adjunct in the management of type 2 diabetes.

Credits

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Conflict of Interest

Robert Lodder was president of Spherix at the time the clinical data were collected.

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Appendix B

Effects of Three Low-Doses of D-Tagatose on Glycemic Control Over Six Months in Subjects with Mild Type 2 Diabetes Mellitus Under Control with Diet and Exercise[1]

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Abstract[1]

The primary objective of this study was to evaluate the safety and the effect of D-tagatose on the glycemic control of subjects with type 2 diabetes as determined by HbA1c levels at the end of 6 months of therapy using the subject's own baseline HbA1c level as a comparator. The determination of the minimal dose required to cause a statistically significant reduction in HbA1c was of particular interest. Eight weeks after screening, the qualifying subjects were randomized to receive one of three doses of D-tagatose: 2.5 g TID, 5.0 g TID or 7.5 g TID. Blood levels of HbA1c, fasting blood glucose concentrations, plasma lipids, changes in body weight, changes in body mass index, and change in insulin levels were checked at each study visit and at the end of the study. Treatment success, as measured by the reduction of HbA1c, was greatest for the 7.5 g D-tagatose dose group, although the difference between the treatments was not statistically significant. For fasting glucose, only the 7.5 g dosage group exhibited reductions from baseline at the 3- and 6-month time points. Mean body weights reduced in a dose-response fashion, with the 5.0 g and the 7.5 g D-tagatose doses providing the greatest reductions. D-tagatose at dosages of 2.5 g, 5.0 g, and 7.5 g TID for six months were well tolerated by this subject population. D-tagatose at 5.0 g TID was the minimal dose required to reduce HbA1c. D-tagatose at 7.5 g TID provided the greatest effect in most measured efficacy parameters.

Introduction[1]

Peripheral insulin resistance and progressive failure of pancreatic β-cell function leading to inadequate insulin secretion are the two principal abnormalities that characterize the pathogenesis of type 2 diabetes.[2] Early intervention at the onset of type 2 diabetes generally consists of maintaining a proper diet and weight, along with regular exercise. If control of blood glucose deteriorates further, then pharmacological intervention with one or more oral anti-diabetic agents is required. Unfortunately for many, type 2 diabetes will continue to progress and exogenous insulin treatment as primary therapy with oral anti-diabetic agents as adjunctive therapy are required in order to achieve glycemic control. Treatment with insulin is usually a point of no return for these patients and a major treatment goal is to prevent progression to this point. Despite all of these interventions including insulin and the introduction of a number of novel agents for the treatment of type 2 diabetes in recent years, glucose control for many remains unsatisfactory.

Recently, a review that included 140 controlled trials and 26 observational studies comparing diabetes medications, both as monotherapy and in two-drug combinations, concluded that there was not enough evidence to clearly support the use of one drug or drug combination over another for stemming the complications of diabetes, including macrovascular and microvascular complications and mortality.[3] Metformin, a drug introduced in the United States in 1994 and as early as 1958 in other countries, is still the most common first drug of choice for the treatment of type 2 diabetes. Compared to the newer drugs, metformin is the drug that has the highest benefit-to-risk ratio for intermediate outcomes, such as HbA1c reduction, less weight gain and less risk of hypoglycemia. However, no currently available therapy has been shown to slow the decline in β-

cell function in established type 2 diabetes. Even with aggressive intervention, it is estimated that 60% of diabetics don't achieve target blood sugar levels with their current treatment.[4] Moreover, particular drugs available for treatment of diabetes may result in unwanted weight gain (long and rapid acting insulin, sulfonylureas, thiazolidinediones, repaglinide, nateglinide), hypoglycemia (insulin, sulfonylureas), gastrointestinal distress (metformin, α-glucosidase inhibitor, amylin mimetics, bile acid sequestrant, bromocriptine), or more serious adverse events such as pancreatitis (short and long-acting glucagon-like peptide-1 (GLP-1) agonists and dipeptidyl peptidase-4 (DDP-4) inhibitors).[5,6] These findings illustrate the limitations of drug therapies currently available for the progression of diabetes.

The observation that many diabetics are not able to consistently control their blood sugar levels within recommended limits using the best available treatments, uncovers the serious need for a drug that can slow and/or halt the progression of diabetes. Preferably, such a drug should exhibit a unique mode of action to enable additive or synergistic use with current therapies; produce no weight gain, hypoglycemia, or other limiting or unmanageable side effects; preserve or enhance β-cell function; and reduce cardiovascular risk factors that lead to morbidity and mortality.

D-tagatose is an isomer of fructose and is ~90% as sweet as sucrose, or sugar. D-tagatose was designated in 2001 as a Generally Recognized as Safe (GRAS) product by the United States Food and Drug Administration and is used as a nutritive or low-calorie sweetener.[7,8] Currently, D-tagatose may be used as a sweetener in diet beverages, light ice creams or yogurts, and regular or dietetic hard candies [Rulis Agency response letter GRAS notice]. Preliminary animal and pre-clinical studies of D-tagatose have demonstrated its ability to lower blood glucose and lipoprotein levels. When consumed, D-tagatose functions as a "sugar blocker" by inhibiting lipid formation from carbohydrates without stimulation of pancreatic beta cells for insulin production or secretion.[8] Regarding lipoprotein levels, D-tagatose has been shown to reduce total cholesterol and VLDL and LDL-cholesterol when compared to sucrose,[9] and increase HDL-cholesterol levels.[10] A number of clinical trials demonstrating the ability of D-tagatose to blunt postprandial rises in blood glucose and reduce HbA1c have been conducted on healthy subjects and diabetic patients.[10–15] Single-dose and repeated-dose studies in healthy and diabetic human subjects have shown that the predominant adverse effects associated with excessive consumption of D-tagatose are gastrointestinal disturbances attributed to osmotic effects from incompletely absorbed D-tagatose.[10–15] Such effects are also commonly associated with excessive consumption of other poorly digestible carbohydrates including polyols. Therefore, D-tagatose shows promise in multiple clinical applications, including the treatment of diabetes. In short, D-tagatose provides glycemic and lipoprotein control through a mechanism of action unlike any agent that is currently available on the market in the United States.

The primary objective of this study was to evaluate the effect of three low-doses of D-tagatose on the glycemic control of subjects with type 2 diabetes. The purpose was to determine the minimum dose able to reduce HbA1c. The secondary objectives were to evaluate the effect of these three doses of D-tagatose compared to the subject's own baseline levels for:

- HbA1c at each study visit
- fasting plasma glucose
- fasting lipid profiles
- insulin concentration
- changes in body weight
- the number of subjects requiring additional anti-diabetic medications and/or withdrawal from the study due to high glycemic measurements

In addition, the safety of D-tagatose in regards to hypoglycemic episodes, gastrointestinal side effects, other adverse events, clinical laboratory abnormalities, and physical examinations were evaluated.

Subjects and Methods[1]

Ethics

The protocol was reviewed and approved by an Institutional Review Board (IRB) before the study was initiated. This trial was conducted in accordance with regulations governing clinical trials including the US Code of Federal Regulations (CFR), Title 21, Part 50; regulations governing IRBs, Title 21, Part 56; and the Declaration of Helsinki concerning medical research in humans. Additional governing regulations included US CFR Title 21, Part 54 and US CFR Title 21, Part 312. This study was also conducted according to International Conference on Harmonization (ICH) Good Clinical Practices (GCP).

Subject Information and Consent

Prior to entry into the study, the nature and risks of the study were reviewed with each subject. Each subject or each subject's legal representative was given the opportunity to read the IRB-approved consent form and to ask questions.

Criteria for Evaluation

The populations analyzed for efficacy endpoints were the intent-to-treat (ITT) population and the efficacy evaluable (EE) population. The population used to analyze safety parameters was identical to the ITT population. Analysis of demographic and baseline for the ITT, EE and Safety populations indicated no gross dissimilarities between the three D-tagatose dose groups. The majority of subjects in each of the treatment groups and populations were male, Asian, non-smoking, non-drinking, around 50 years of age, weighing approximately 150 lbs, with diet and exercise for control of their diabetes. Usage of allowable medications during the trial was also similar across the three treatment groups.

Primary Efficacy Endpoint—The primary endpoint for this Phase 2 dose-ranging study was a decrease of ≥ 0.5% in HbA1c level after 6 months of the study treatment.

Secondary Efficacy Endpoints—Secondary endpoints for this study included assessment of the effects of D-tagatose on other glycemic control measurements. These measurements included the proportions of subjects at each visit who achieved a decrease in HbA1c of ≥ 0.5%; proportions of subjects at each visit who achieved a decrease in HbA1c level of ≥ 1%; change from baseline to each study visit in fasting blood glucose concentrations, and plasma lipids (triglycerides, low density lipoprotein, total cholesterol, and high density lipoprotein); changes in body weight; changes in body mass index; and change in insulin levels.

Safety—Descriptive statistics and by-subject data listings were prepared for all safety parameters. No inferential statistics were performed for safety parameters. Safety was assessed through the entire duration of the study, and in the event of an adverse event (AE) or serious adverse event (SAE), safety was to be monitored until the AE/SAE resolved, or until the AE/SAE was deemed chronic or stable by the investigator.

Study Design and Plan

This study was designed as a prospective, randomized, 6-month, parallel dose-ranging trial in subjects with mild type 2 diabetes who, at the time of randomization, were not taking any oral

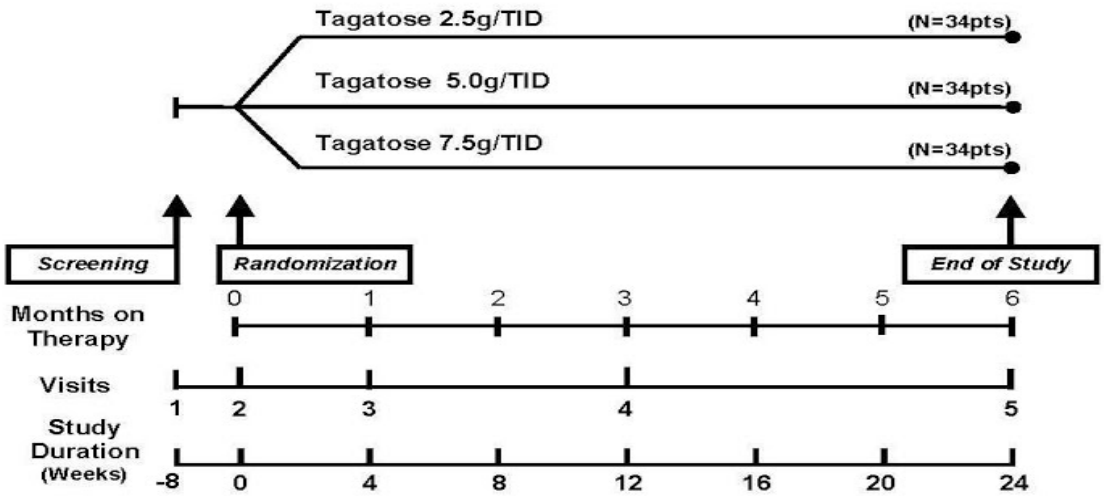
anti-diabetic or anti-hyperglycemic medication or parenteral anti-diabetic medication and were controlling their diabetes with diet and exercise alone. This Phase 2 parallel dose-ranging trial was designed to evaluate the dose-response effect of minimal doses of D-tagatose (2.5, 5.0, or 7.5 g, TID) on glycemic control in subjects with type 2 diabetes, who had undergone an 8-week run-in period of standardized diet and exercise. This 8-week stabilization period was considered a sufficient duration of standardized diet and exercise to provide a homogenous population of subjects with type 2 diabetes. No active control group was utilized in the design as the study was intended as a dose-ranging trial. The data from this trial was not intended to provide evidence of equivalence or superiority to any currently marketed medication.

A total of 112 subjects were planned to be randomized into the study (i.e., 34 subjects to each of three treatment groups: 2.5 g, 5.0 g, and 7.5 g D-tagatose given orally, three times daily, immediately prior to meals). Eight weeks after screening and stabilization, qualified subjects were randomized to receive one of three doses of D-tagatose. All doses of D-tagatose were premixed with drinking water into a solution of 4 ounces per dose. The study design is depicted in Figure 1.

At the initial visit (Visit 1) subjects were screened for eligibility for entering the study. Subjects who were eligible were those diabetic subjects treated solely with diet and exercise and who had mildly elevated HbA1c levels but were otherwise in good health and were not suffering from any serious complications of diabetes or any other significant concurrent disease. Subjects were not to be taking any medications for the treatment of type 2 diabetes. The screening visit consisted of each subject undergoing a review of their relevant medical history and a physical examination, both of these primarily aimed at finding any abnormalities related to complications of diabetes. Additionally, clinical laboratory testing (including comprehensive hematology, clinical chemistry, liver function tests, lipid profile, HbA1c levels and urinalysis) was performed. At the end of the screening visit, potentially eligible study subjects were instructed to follow a weight-maintaining diet and a daily exercise program under the supervision of the investigator. They were given a blank subject diary and a nutritional diary, and were scheduled for the second visit (Visit 2) after 8 weeks of stabilization. No study drug was distributed at the screening visit (Visit 1).

Figure 1[1]

Study Schedule



Subjects underwent an 8-week run-in period of standardized diet and exercise to provide a homogenous population of subjects with type 2 diabetes prior to randomization into three treatment groups. Treatment lasted for 6 months. Total duration of the study for subjects was 24 weeks with five study visits during the trial.[1]

All subjects who were eligible were randomized during Visit 2 which took place within 8 weeks (± 7 days) of Visit 1. This was a single-blind study, in which subjects were blinded to dose group. At Visit 2, prior to randomization, baseline procedures including a complete medical evaluation with a review of medical history changes since Visit 1 and a physical examination were performed on qualifying subjects. In addition, subjects had blood drawn for clinical laboratory testing and urinalysis.

Table 1[1]

Schedule of Events

	Visit 1 Screening	Visit 2 Randomization	Visit 3 Month 1	Visit 4 Month 3	Visit 5 End of Study
Subject Registration to Study	X				
Inclusion and Exclusion Criteria	X				
Informed Consent*	X				
Physical Examination	X	X			X
Medical History/Update	X	X	X	X	X
Record Concomitant Medications	X	X	X	X	X
Record Adverse Events		X	X	X	X
Hematology Panel	X	X	X	X	X
Chemistry Panel†	X	X	X	X	X
Liver Function Tests‡	X	X	X	X	X
Lipid Profile§	X	X	X	X	X
HbA_{1c} levels	X	X	X	X	X
Urinalysis Panel	X	X	X	X	X
Dispense Study Drug		X	X	X	
Dispense diaries	X	X	X	X	
Compensation	X	X	X	X	X
Collect empty study drug vials/packages			X	X	X
Collect completed subject diaries		X	X	X	X

* Explained to and signed by subject

† SMA-18 (or equivalent), creatinine clearance, and insulin levels

‡ Alanine transaminase (ALT), aspartate aminotransferase (AST), total bilirubin, alkaline phosphatase, total protein, and albumin

§ Total cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), and triglycerides[1]

Randomization was stratified by site and baseline HbA1c levels (< 7.5% or ≥ 7.5%) to obtain a balanced distribution of subjects across the three arms of the trial. In addition to a supply of study medication, subjects received a diary and diary completion instructions for recording side effects, intercurrent illnesses/symptoms, and concomitant medications.

For Visits 3 and 4, subjects returned to the clinic for diary assessment, blood tests, and study drug compliance assessment and dispensation of additional study drug. Each of these visits had a ± 7 day window. Subjects had blood drawn for clinical laboratory testing and urinalysis.

During the final visit (Visit 5), the diary and all used and unused medication packets were collected and all assessments conducted at Visits 3 and 4 were repeated. Additionally, a final physical examination was conducted. Subjects were instructed to return to their primary physician for subsequent diabetes care and follow-up. This visit had a ± 7 day window. The efficacy and safety measurements that were evaluated throughout the study are provided in the study schedule (Table 1).

In the event a subject was discontinued for any reason, an attempt was to be made to keep the subject on the safety arm of the study. Study treatment for discontinued subjects was to be stopped but the subject was to continue with the protocol assigned study visits but not the interim study drug supply visits. In the event the subject declined participation in the safety arm of the trial, after treatment discontinuation, Visit 5 (the End of Study Visit) was to be scheduled at least 30 days after the last dose.

Clinical Samples

Blood samples used to assay HbA1c, blood glucose, insulin concentrations and lipids (total cholesterol, triglycerides, HDL, and LDL) were taken at each study visit, including the screening visit prior to the run-in period. All samples were collected and processed at the study center and then forwarded to the central laboratory (ICON Central Laboratories, Inc., Farmingdale, NY), by overnight courier, for assay.

Statistical Method

Three analysis populations were evaluated:

Intent-to-Treat (ITT) Population—The main efficacy analysis was conducted using the ITT Population. The ITT population included all subjects who had signed the study Informed Consent Form, received the protocol-specified treatment, and had a baseline and at least one post-baseline HbA1c value.

Efficacy Evaluable (EE) Population—The EE population was used for supportive analyses. The EE population consisted of all randomized subjects who completed treatment periods, received at least 80% of the study medication, and had no major protocol violations or eligibility violations. All protocol violations were to be identified prior to database lock.

Safety Population—The safety population was used for the analysis of safety variables. The safety population consisted of all randomized subjects who received at least one dose of study medication and had at least one post treatment visit of safety assessment.

Determination of Sample Size

The sample size calculation was based on a reduction of at least 0.5% in HbA1c level after 6 months of the study treatment compared to baseline for each dose group, a standard deviation of 1.0 for each treatment group, and an 80% statistical power with a two-sided analysis at a Type

I error rate of 0.05. The required number of evaluable subjects was about 102 (34 for each D-tagatose dose group) based on nQuery Advisor, version 6.01. A total of 40 subjects per treatment group (120 subjects for the study) were to be recruited, as it was expected to observe a 15% drop out rate in this study population, and a total of 150 subjects (50 for per treatment group) was screened based on the estimated screen failure rate of 20%.

Descriptive statistics and by-subject data listings were prepared for all efficacy parameters. For continuous data, summaries included number of observations, mean, standard deviation, median, minimum, and maximum values. For categorical data, summaries included frequency counts and percentages. All statistical tests used in efficacy assessments were 2-sided, with no p-value adjustment. Data are presented as the mean ± standard deviation.

The primary efficacy variable was change in HbA1c level from baseline to 6 months. Baseline was defined as the last value obtained prior to the first randomized treatment. Changes from baseline HbA1c level were assessed using the general linear model (ANCOVA) to adjust for baseline differences and the stratification factor. Factors in the model included treatment and stratum of baseline HbA1c (< 7.5% or ≥ 7.5%). The least square means and standard error were derived from the general linear model for each dose group.

For secondary efficacy endpoints, (1) logistic regression was used to investigate the effect of treatment on the endpoint (i.e., decrease of ≥ 0.5% HbA1c reduction at each visit) and, decrease of ≥ 0.5% HbA1c reduction at each visit, and (2) analysis of covariance (with stratification factor as covariate) was used to compare changes from baseline in fasting blood glucose, triglycerides, low density lipoprotein, total cholesterol, high density lipoprotein, body weight, body mass index, and insulin level.

Results[1]

Study Population

The study evaluated the data from 18 clinical study sites (11 sites in the United States and 7 sites in India) and 161 subjects were randomized to one of the three D-tagatose dosages (2.5 g: n = 57, 5.0 g: n = 51, and 7.5 g: n = 53). Of the 161 randomized subjects, 60 (37%) were withdrawn before completing the 6-month treatment period. The most common reasons for withdrawal were subject request (18/161, 11%) and subject lost to follow up (11/161, 7%). The populations analyzed for efficacy endpoints were the ITT population (145 total; 52 in the 2.5 g treatment group, 46 in the 5.0 g treatment group and 47 in the 7.5 g treatment group) and the EE population (87 total, 31 in the 2.5 g treatment group, 29 in the 5.0 g treatment group, and 27 in the 7.5 g treatment group). The population used to analyze safety parameters was identical to the ITT population. There were no indicators of dissimilarity between the treatments in any of the analysis populations in terms of demographic or baseline characteristics, concomitant medication usage, or compliance with treatment, diet, or exercise.

Efficacy Results

Incidence of ≥ 0.5 HbA1c decrease after six months of treatment—Treatment success, as measured by the incidence of a 0.5 minimum HbA1c decrease from baseline after six months of treatment, was greatest for the 7.5 g TID dose of D-tagatose in both the ITT (25%, 12/47) and the EE (33%, 9/27) populations (see Figure 2). Treatment success, as measured by the primary endpoint (a reduction from baseline of 0.5 in HbA1c after six months of treatment) was greatest for the 7.5 g D-tagatose dose group, although the difference between the treatments was not statistically significant. For the 7.5 g dose group 25% of the population achieved this treatment

success parameter compared to 19% in the 2.5 g dose group and 15% in the 5.0 g dose group. The difference across the three dose groups was not statistically significant for either of the analysis populations ($p > 0.05$, logistic regression).

Incidence of ≥ 0.5 HbA1c decrease after one, three and six months of treatment—Incidence summaries of ≥ 0.5 HbA1c reduction from baseline after one, three, and six months of treatment for each of the D-tagatose dose groups are provided in Table 2 for the ITT population and in Table 3 for the EE population. Only the 7.5 g D-tagatose dose group indicated a dose-response trend over time; i.e., for the ITT population, treatment success was indicated for 4/47 (8%) after one month of treatment, for 5/47 (11%) after three months of treatment, and 12/47 (25%) after six months of treatment. For the EE population, success was indicated for 2/27 (7%) at the one and three month treatment time points and for 9/27 (33%) subjects at the six month time point. By logistic regression, the incidences across the three treatments at a single time point were not statistically significantly different.

Figure 2[1]

Incidence of ≥ 0.5 HbA_{1c} Decrease After Six Months of Treatment, ITT and EE Populations

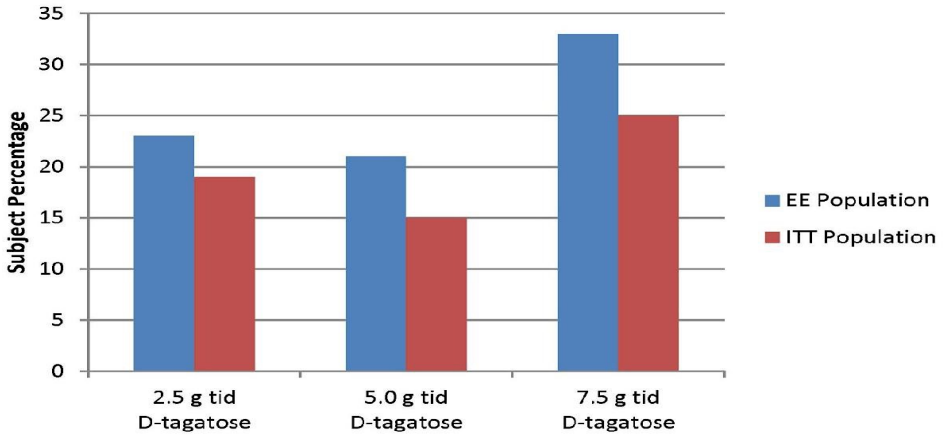


Table 2[1]

Incidence of ≥ 0.5 HbA_{1c} Decrease from Baseline After One, Three, and Six Months of Treatment, ITT population

	D-tagatose 2.5g	D-tagatose 5.0g	D-tagatose 7.5g
Treatment Duration	**N = 51 n (%)**	**N = 46 n (%)**	**N = 47 n (%)**
One Month	8 (15.38%)	9 (19.57%)	4 (8.51%)
Three Months	11 (21.15%)	8 (17.39%)	5 (10.64%)
Six Months	10 (19.23%)	7 (15.22%)	12 (25.53%)

N = denominator for all percentages[1]

Table 3[1]

Incidence of ≥ 0.5 HbA_{1c} Decrease from Baseline After One, Three, and Six Months of Treatment, EE Population

	D-tagatose 2.5g	D-tagatose 5.0g	D-tagatose 7.5g
Treatment Duration	**N = 31 n (%)**	**N = 29 n (%)**	**N = 27 n (%)**
One Month	6 (19.35%)	7 (24.14%)	2 (7.41%)
Three Months	9 (29.03%)	8 (27.59%)	2 (7.41%)
Six Months	7 (22.58%)	6 (20.69%)	9 (33.33%)

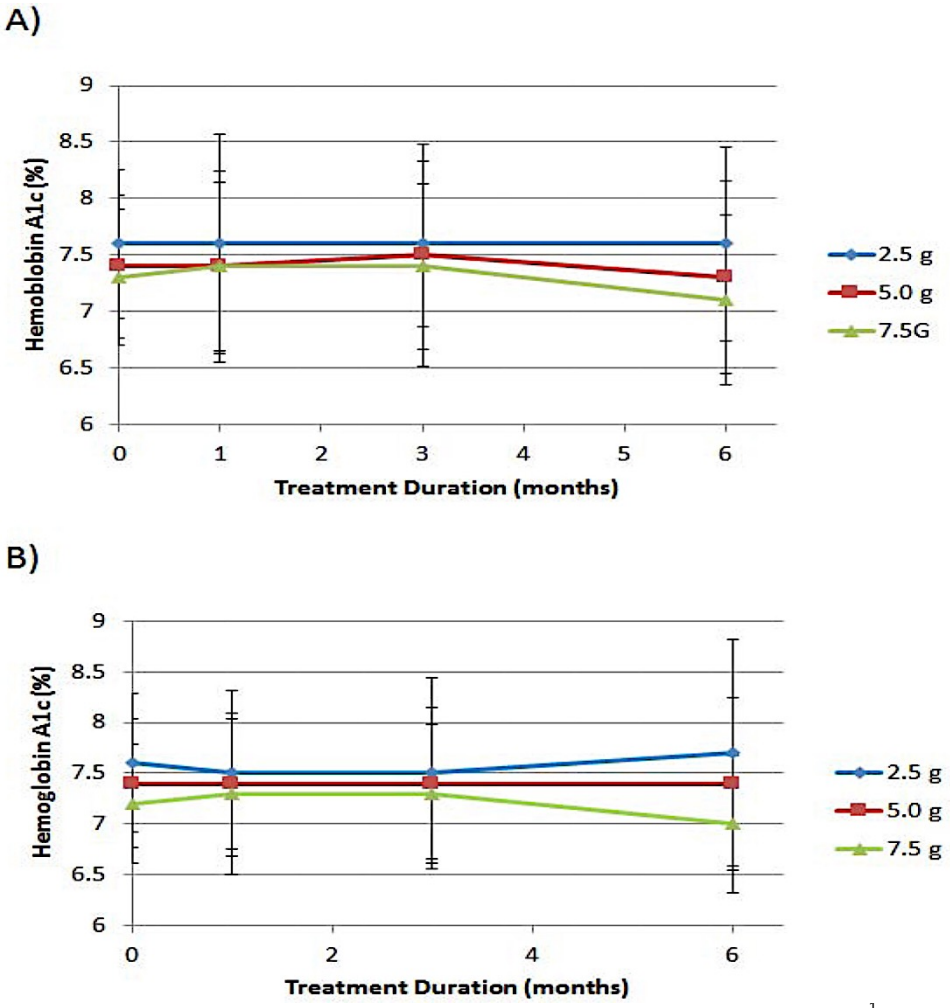
N = denominator for all percentages[1]

Effect of D-tagatose on HbA1c values—The mean HbA1c levels for the three D-tagatose groups after 1, 3, and 6 months of treatment for the ITT and the EE populations are depicted in Figures 3A and 3B, respectively. For both analysis populations, a dose-response trend was observed for the 5.0 g and 7.5 g D-tagatose doses after 3 months and 6 months of treatment. Statistically, significant differences between the doses, however, were not achieved at any of the post-treatment time points ($p > 0.5$, ANCOVA with baseline as covariate).

Reductions from baseline to six months in mean HbA1c were observed for the 5.0 g and the 7.5 g dose groups but not for the 2.5 g dose group; i.e., mean baseline and 6-month values were 7.6 ± 0.66% and 7.6 ± 1.01%, respectively, for the 2.5 g dose group; 7.4 ± 0.63% and 7.3 ± 0.85%, respectively, for the 5.0 g dose group; and 7.3 ± 0.60% and 7.1 ± 0.75%, respectively, for the 7.5 g dose group (Figure 3B). These reductions indicate a positive dose response, although the differences in the observed mean reductions were not statistically significant between the three D-tagatose doses.

Figure 3[1]

Effect of D-tagatose on Mean HbA1c Values After 1, 3, and 6 Months of Treatment

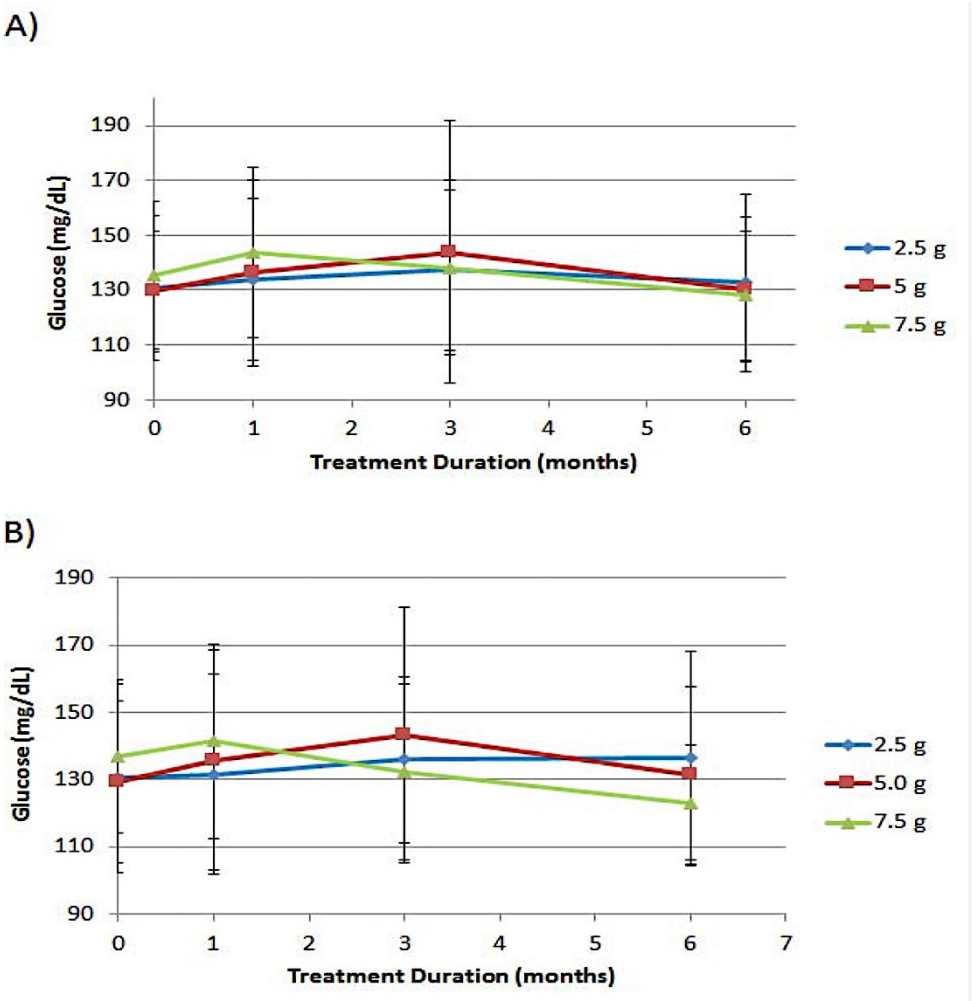


ITT population (A) and EE population (B). Data are presented as the mean ± standard deviation.[1]

Effect of D-tagatose on fasting glucose values—For fasting glucose, only the 7.5 g dosage group exhibited reductions from baseline at the 3- and 6-month time points in both analyzed populations (ITT and EE, Figures 4A and 4B, respectively). For the EE population, baseline and 6-month means in fasting glucose were 130.4 ± 28.8 mg/dL and 136.3 ± 31.9 mg/dL, respectively, for the 2.5 g treatment; 129.5 ± 24.0 mg/dL and 131.2 ± 26.3 mg/ dL, respectively, for the 5.0 g treatment; and 136.7 ± 22.8 mg/dL and 123.1 ± 17.1 mg/dL, respectively, for the 7.5 g treatment ($p = 0.0268$) (Figure 4B). For the ITT and the EE populations, the 7.5 g D-tagatose dose group provided a mean reduction in fasting glucose level after six months of treatment.

Figure 4[1]

Effect of D-tagatose on Mean Fasting Glucose Values After 1, 3, and 6 Months of Treatment

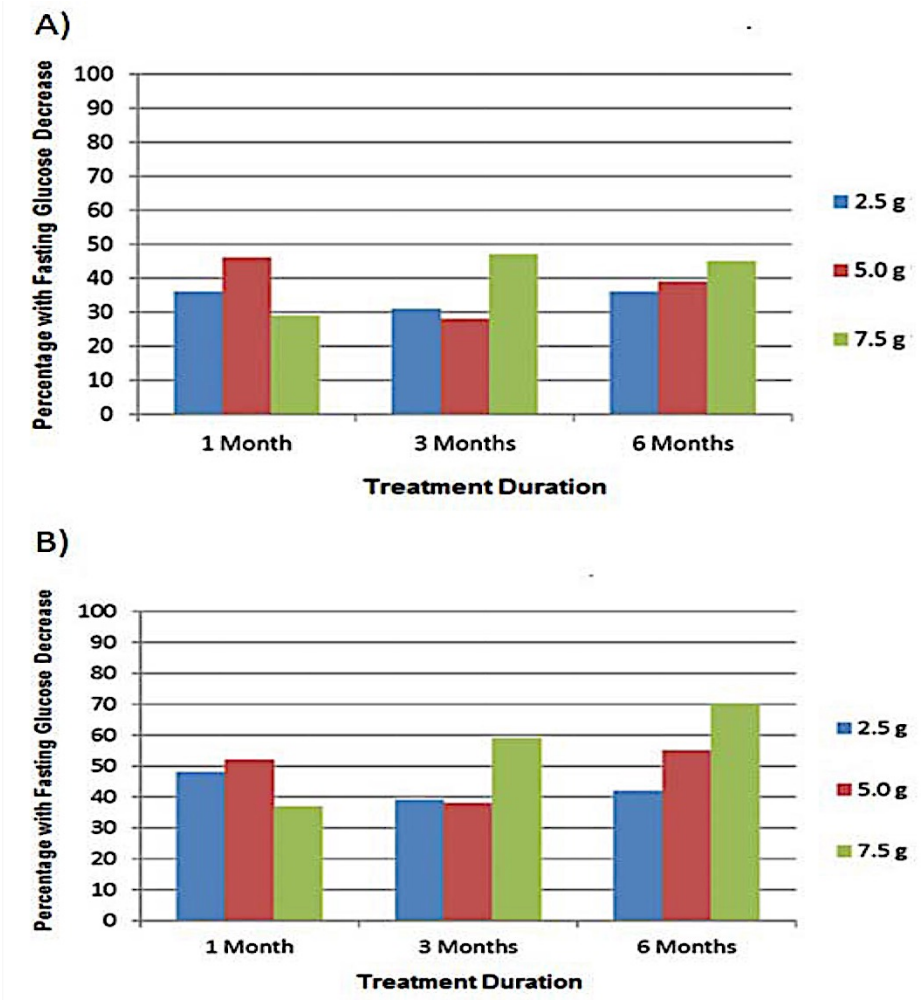


ITT (A) and EE (B) populations. Data are presented as the mean ± standard deviation.[1]

Incidence of decreases in fasting glucose levels—Results indicate a dose-response trend for the incidence of fasting glucose decreases at 6 months in both the ITT and the EE populations. For the ITT population, the incidences of fasting glucose decreases after six months of treatment were 45% (21/47), 39% (18/46), and 36% (19/52) for the 7.5 g, 5.0 g, and 2.5 g D-tagatose treatment groups, respectively. For the EE population, the incidences of glucose decreases were 70% (19/27), 55% (16/29), and 42% (13/31), respectively. These data are depicted in Figure 5. No statistically significant differences across the treatments ($p>0.5$, logistic regression) were observed for either population at any of the post treatment time points.

Figure 5[1]

Effect of D-tagatose on the Incidence of Fasting Blood Sugar (i.e., Glucose) Decreases After 1, 3, and 6 Months of Treatment

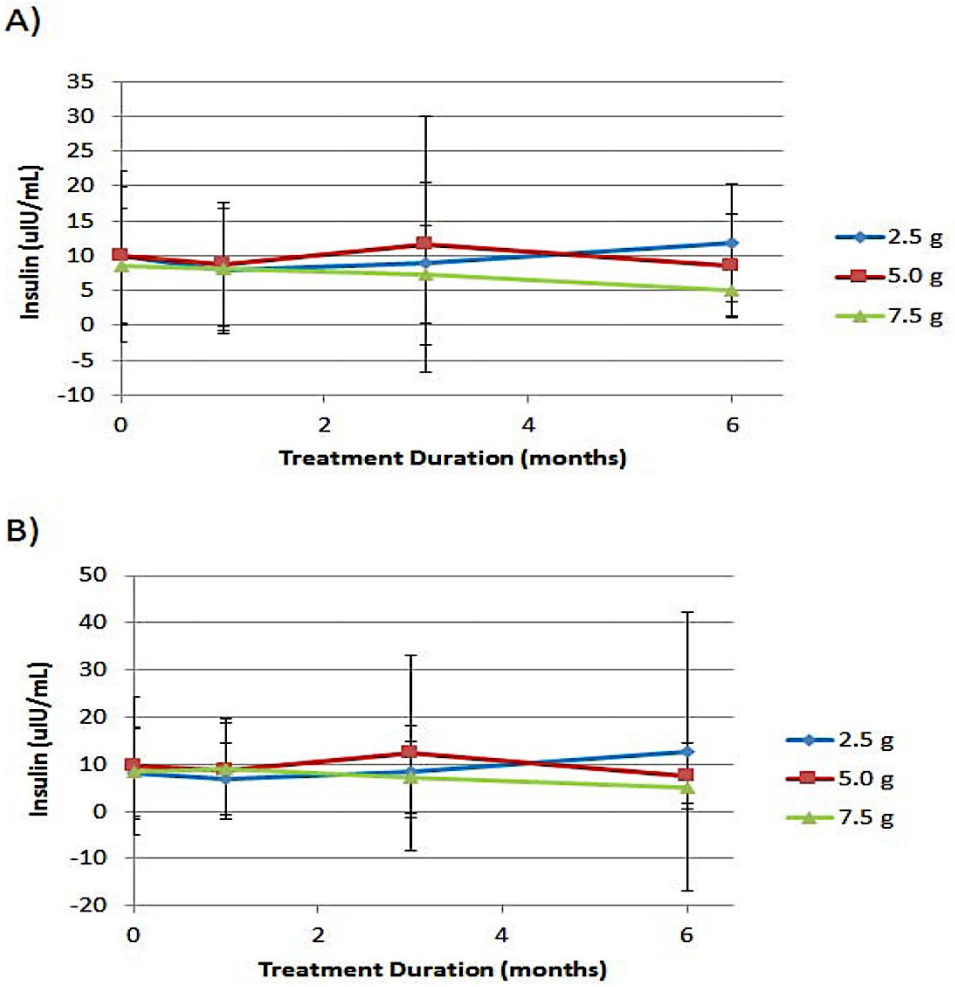


ITT (A) and EE (B) populations.[1]

Effect of D-tagatose on serum insulin—The mean insulin levels (µIU/mL) for the three D-tagatose dose groups after 1, 3 and 6 months of treatment and the two analysis populations are depicted in Figure 6. For both analysis populations, the 7.5 g D-tagatose dose group showed consistent reductions from the mean baseline insulin level after three and six months of treatment. For the ITT population (Figure 6A), the 7.5 g D-tagatose dose group demonstrated a mean baseline insulin level of 8.5 ± 8.2 µIU/mL; and after three and six months of treatment the mean insulin levels for this dose group were 7.3 ± 7.0 µIU/mL and 5.0 ± 3.6 µIU/mL, respectively. For the EE population (Figure 6B), the 7.5 g D-tagatose dose group demonstrated a mean baseline insulin level of 9.1 ± 10.6 µIU/mL; and after three and six months of treatment the mean insulin levels for this dose group were 7.2 ± 7.7 µIU/mL and 5.1 ± 3.4 µIU/mL, respectively. Statistically significant differences between the doses, however, were not achieved at any of the post-treatment time points ($p > 0.5$, ANCOVA with baseline as covariate) for either the ITT or the EE populations.

Figure 6[1]

Effect of D-tagatose on Mean Insulin Values After 1, 3, and 6 Months of Treatment

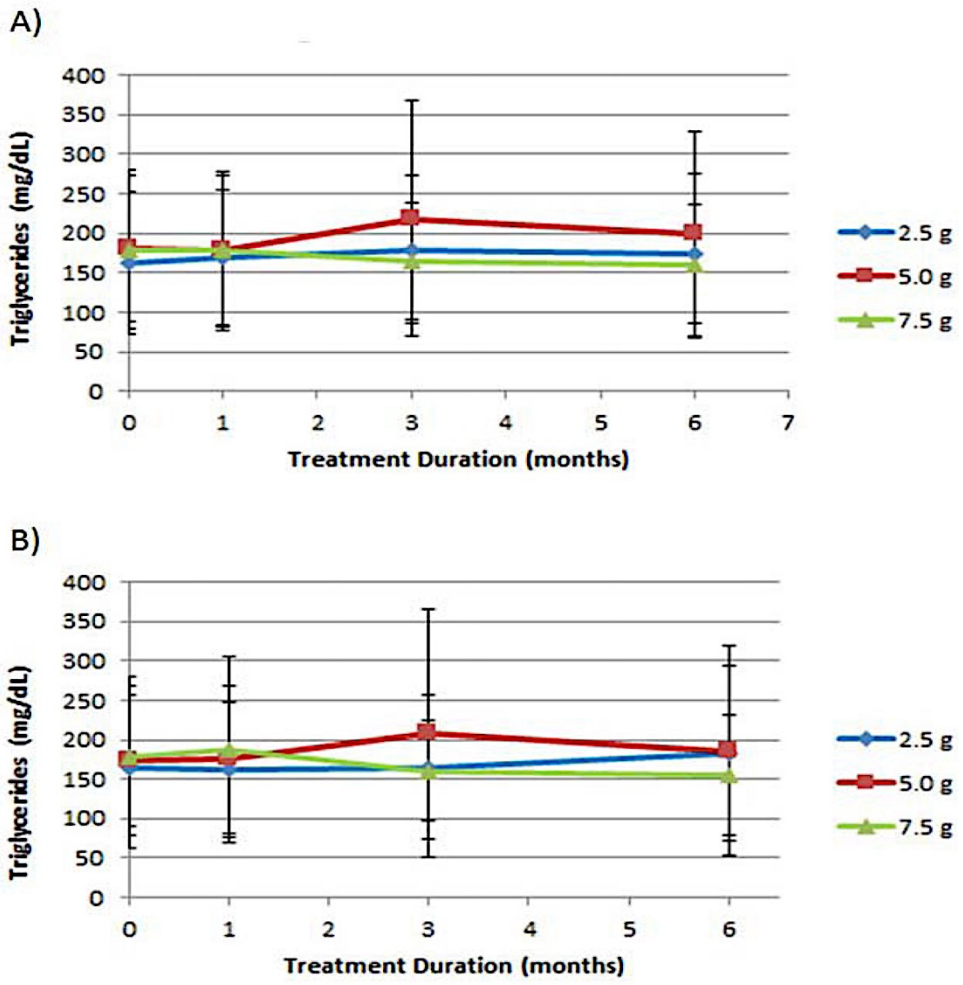


ITT (A) and EE (B) populations. Data are presented as the mean ± standard deviation.[1]

Effect of D-tagatose on lipids—No statistically significant differences between treatments at any measured time point were observed for total, LDL, or HDL cholesterol measurements (data not shown); and all dosages provided some measure of reduction from mean baseline values at one or more time points. There was a striking elevation in mean triglyceride level after three months of treatment with the 5.0 g D-tagatose dose group in both ITT and EE populations (Figure 7). For the ITT population, the mean triglyceride concentrations at baseline and three months for the 5.0 g dose were 181 ± 92.7 mg/dL and 218 ± 149 mg/dL, respectively; 162 ± 89.8 mg/dL and 179 ± 93.4 mg/dL, respectively, for the 2.5 g dose; and 179 ± 99.8 mg/dL and 165 ± 74.7 mg/dL, respectively, for the 7.5 g dose. The difference between the treatment groups at the 3-month time point was statistically significant in the ITT population ($p = 0.0296$, ANCOVA with baseline as covariate). Similar results were observed with the EE population; however, the difference between treatments at three months was not statistically significant with the EE population.

Figure 7[1]

Effect of D-tagatose on Mean Triglyceride Values After 1, 3, and 6 Months of Treatment

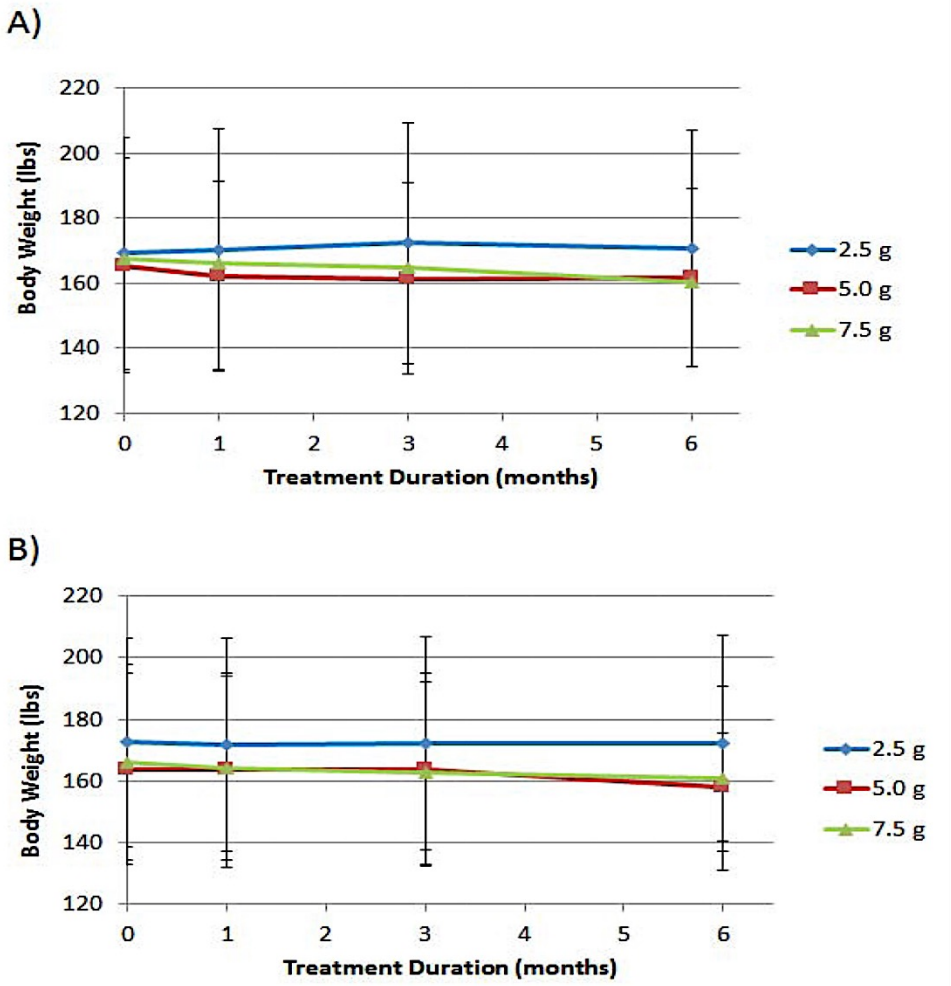


ITT (A) and EE (B) populations. Data are presented as the mean ± standard deviation.[1]

Effect of D-tagatose on body weight—Mean body weights, over time, reduced in a dose-response fashion, with the 5.0 g and the 7.5 g D-tagatose doses providing the greatest reductions (Figure 8). Mean body weights at baseline, 3-months, and 6-months for the 5.0 g D-tagatose dose group were 165.4 ± 33.0 lbs, 161.5 ± 29.5 lbs, and 161.7 ± 27.5 lbs, respectively; and the mean body weights for the 7.5 g D-tagatose dose group were 167.4 ± 33.0 lbs, 165.1 ± 30.5 lbs, and 160.6 ± 29.3 lbs, respectively. The mean body weights for the 2.5 g D-tagatose dose group and the EE population remained within 1 lb of mean baseline weight at all post baseline time points. There was a statistically significant difference between the mean weights of the three treatments at the 3-month time point for the EE population ($p = 0.0345$, ANCOVA with baseline as covariate).

Figure 8[1]

Effect of D-tagatose on Mean Body Weight After 1, 3, and 6 Months of Treatment



ITT (A) and EE (B) populations. Data are presented as the mean ± standard deviation.[1]

Safety results—D-tagatose at all doses was well-tolerated. None of the reported adverse events were unexpected and the majority was of a gastrointestinal nature, as expected. The incidences of all reported events were similar for all treatments. There were no disparities between the three treatments in any of the assessed safety parameters. Nearly all reported AEs were of mild or moderate severity. Severe AEs occurring during the study are summarized in Table 4. There did not appear to be a dose-response relationship in terms of AE severity. As expected, the highest incidences of probably or possibly related AEs were gastrointestinal disorders. No dose-response trends were observed for any of the AEs that were assessed as possibly or probably related to study treatment.

Table 4[1]

Incidence of Severe Adverse Events, Safety Population

	D-tagatose 2.5g N = 52 n (%)	D-tagatose 5.0g N = 46 n (%)	D-tagatose 7.5g N = 47 n (%)	Total N = 145 n (%)
Nausea	-	-	1 (2.1%)	1 (0.7%)
Retching	-	-	1 (2.1%)	1 (0.7%)
Vomiting	1 (1.9%)	-	-	1 (0.7%)
Fatigue	-	1 (2.2%)	-	1 (0.7%)
Lethargy	-	1 (2.2%)	-	1 (0.7%)
Anxiety	1 (1.9%)	-	-	1 (0.7%)

Disposition of subjects—The study disposition of randomized subjects is provided in Table 5. One hundred and sixty one (161) subjects were randomized and analyzed. Of the 161 randomized subjects, 57/161 (35%) were randomized to the 2.5 g dose group, 51/161 (32%) were randomized to the 5.0 g dose group, and 53/161 (33%) were randomized to the 7.5 g dose group.

Table 5[1]

Study Disposition of Randomized Subjects

	D-tagatose 2.5g	***D-tagatose 5.0g***	***D-tagatose 7.5g***	***Total***
Number Randomized (N)*	*57*	*51*	*53*	*161*
	n (%)	***n (%)***	***n (%)***	***n (%)***
Received ≥ 1 Dose	*55 (96.5%)*	*49 (96.1%)*	*51 (96.2%)*	*155 (96.3%)*
Completed Study†	*35 (61.4%)*	*32 (62.7%)*	*34 (64.2%)*	*101 (62.7%)*
Withdrawn	*22 (38.6%)*	*19 (37.3%)*	*19 (35.8%)*	*60 (37.3%)*

**Denominator for all percentages.*

†Completed study through the end of the 6-month treatment period.[1]

All treatment groups had a median study medication compliance of approximately 80%, and full to good compliance with the study-mandated diet and exercise, as assessed by the investigator, was 77% to 87% after the first month, 70% to 79% after three months, and 63% to 74% after six months. Given the similarity of the treatment groups in terms of demographics, baseline characteristics, concomitant medication usage, and compliance with treatment, diet and exercise, the resulting efficacy and safety analyses were considered reliable for comparisons between three D-tagatose dose groups.

Sixty (60) randomized subjects (37.3%) were withdrawn before completing the 6-month treatment period. Two subjects, from each of the dose groups were withdrawn prior to receiving the first dose of study medication, resulting in a similar withdrawal rate for each of the three doses. However, when individual reasons for withdrawal were evaluated, two interesting dose-response trends were noted: one for subject-initiated withdrawals and one for withdrawals due to AEs/SAEs (Table 6).

Table 6[1]

Reasons for Withdrawal, Randomized Subjects

Reason for Withdrawal	D-tagatose 2.5g N = 57 n (%)	D-tagatose 5.0g N = 51 n (%)	D-tagatose 7.5g N = 53 n (%)	Total N = 161 n (%)
Subject Request	3 (5.3%)	5 (9.8%)	10 (18.9%)	18 (11.2%)
Lost to Follow-up	3 (5.3%)	3 (5.9%)	5 (9.4%)	11 (6.8%)
AE/SAE	5 (8.8%)	2 (3.9%)	1 (1.9%)	8 (5.0%)
Protocol Deviation	3 (5.3%)	2 (3.9%)*	2 (3.8%)	7 (4.3%)
Termination by Sponsor	2 (3.5%)	0 (0.0%)	0 (0.0%)	2 (1.2%)
Other	6 (10.5%)†	7 13.7%)	1 (1.9%)	14 (8.7%)
Total Withdrawn	22 (38.6%)	19 (37.3%)	19 (35.8%)	60 (37.3%)

N = Randomized subjects; denominator for all percentages.

*One randomized subject in this dose group had a recorded reason for withdrawal of "screen failure" and was therefore tabulated with those withdrawn for a protocol deviation.

†One randomized subject in this dose group had no reason for withdrawal specified on the CRF and was therefore tabulated with "Other".[1]

Subject-initiated withdrawals—Of the randomized subjects, 18.0% (29/161) were withdrawn due to subject request or lost to follow up. The highest incidence for these two reasons, combined, was with the 7.5 g dose group; i.e., 6/57 (10.5%) in the 2.5 g dose group, 8/51 (15.7%) in the 5.0 g dose group, and 15/53 (28.3%) in the 7.5 g dose group (Table 6). These data suggest that as the dosage increased so did the drop-outs due to "subject request" and "lost to follow-up," combined (i.e., subject-initiated withdrawals). This observation is supported by the fact that, when the two reasons (subject request and lost to follow-up) were analyzed separately, each of the responses also indicated a positive dose-response. These results all appear to indicate that as the dosage increased, so did the incidence of subject-initiated withdrawals.

Withdrawals due to AEs/SAEs—The incidence of withdrawals due to AEs and SAEs for all dose groups combined was 8/161 (5.0%) with what appears to be an inverse dose-response relationship; i.e., as the dosage increased the incidence of withdrawals due to AEs decreased. The highest incidence of withdrawals due to AEs was in the 2.5 g dose group (5/57, 8.8%) and the lowest incidence of withdrawals due to AEs was in the 7.5 g group (1/53, 1.9%) (Table 6). Withdrawals due to protocol deviations were less than 5% for all doses, combined. The individual dose groups all had similar incidences, ranging from approximately 4% to approximately 5%.

Discussion[1]

This dose-ranging trial was conducted to evaluate the effect of three doses of D-tagatose (2.5 g, 5.0 g, and 7.5 g), taken three times daily over a period of six months, on various glycemic control measures and safety parameters in type 2 diabetic patients. In this trial, the 5.0 g TID dose of D-tagatose was assumed (based on previous trials)[8] to be a minimally effective dose in reducing HbA1c, a primary measurement of glycemic control. The 2.5 g TID dose of D-tagatose (which served as a nominal-effect comparator) and the 7.5 g TID dose were selected to bracket the 5.0 g TID dosage level.

The efficacy parameters selected for evaluation in this trial are all common measures of glycemic control for diabetic patients: HbA1c, fasting glucose levels, lipid parameters, blood insulin levels, and body weight. The primary efficacy parameter selected for this trial was a dichotomous variable: the treatment success as measured by a reduction from baseline HbA1c by at least 0.5

units after six months of treatment (i.e., 0.5% reduction in HbA1c after six months of treatment). Dichotomous variables, by nature, are not the most sensitive metric by which to assess treatment differences; nevertheless, a treatment difference was noted in that 12/47 (26%) of the 7.5 g D-tagatose dose group (ITT population) and 9/27 (33%) EE population in the dose groups met the endpoint (Tables 2 and 3). For both populations, of the three studied dosages, the 7.5 g D-tagatose dose exhibited the greatest incidence of success, as measured by this parameter. Interestingly, a dose response was not observed in the primary endpoint. For the ITT population and the primary endpoint, the 2.5 g and the 5.0 g D-tagatose doses had a success incidences of 10/52 (19%) and 7/46 (15%), respectively and the difference between the three dosages were not statistically significant. Similar results for the lower doses were observed with the EE population. Continuous variables are traditionally more sensitive in detecting differences between treatments. Mean HbA1c reductions after six months of treatment within the ITT population were greatest for the 7.5 g D-tagatose group (Figure 3A). The minimum dosage at which HbA1c reductions were evident was the 5.0 g TID dosage. The mean baseline and the mean 6-month HbA1c values for the 2.5 g treatment group remained the same. The differences between the treatment groups in HbA1c measurements were not statistically significant at any post treatment time point. Similar results with HbA1c reduction were observed with the EE population (Figure 3B).

Regarding fasting glucose mean values and mean changes from baseline over time, a reduction from baseline was observed only in the 7.5 g D-tagatose dose group, beginning after three months of treatment with increasing reduction at the 6-month time point, at which time statistical significance was observed (Figure 4), thereby supporting the analysis of the primary endpoint. Additionally, for the EE population, the incidence rates of subjects achieving any decrease in fasting glucose values indicated consistent increases throughout the treatment period for the 7.5 g dose group, although the differences across the groups was not statistically significant at any time point. This observation provided further support of the primary endpoint results. The observed increases and reductions in fasting glucose levels over time for the 2.5 g and the 5.0 g treatments are most likely due to the inherent variability of the fasting glucose parameter.

Each of the doses had some measure of mean insulin reduction at one or more post-baseline time points in both analyzed populations. After six months of treatment, however, only the 5.0 g and the 7.5 g dosages had mean levels that were lower than that of baseline (Figure 6A). There were no statistically significant differences between treatments at any of the measured time points. Similar results were observed with the EE population (Figure 6B).

No statistically significant difference between treatments at any measured time point was observed for total, LDL, or HDL cholesterol measurements; and all dosages provided some measure of reduction from mean baseline values at one or more time points. There was a striking elevation in mean triglyceride level after three months of treatment with the 5.0 g D-tagatose dose group in both the ITT and the EE populations (Figure 7). This rise in triglycerides at the 5.0 g dose warrants further research.

Mean body weights, over time, were reduced in a positive dose-response fashion, with the 5.0 g and the 7.5 g D-tagatose doses providing the greatest reductions (Figure 8). The mean body weights for the 2.5 g D-tagatose dose group remained within 1 lb of mean baseline weight at all post baseline time points.

D-tagatose at all doses was well tolerated. None of the reported adverse events were unexpected and the majority was of a gastrointestinal nature, as expected. The incidences of all reported events were similar for all treatments. Finally, there were no disparities between the three treatments in any of the assessed safety parameters.

Although not always statistically significant, the high dose (7.5 g TID of D-tagatose) appeared to provide the greatest efficacy of the three tested doses in terms of incidence rates achieving ≥ 0.5% decrease in HbA1c, reductions in fasting glucose values, reductions in lipid parameters, reduction in insulin concentration, and reduction in body weight. Additionally, it appears that 5.0 g TID of D-tagatose was the minimally effective dose for providing reduction in glycemic measures within this type 2 diabetic population.

Future research might investigate the inverse dose-response relationships for constipation, nausea, headache, vomiting, and eructation (Table 6).

Conclusions[1]

Several points can be concluded from this study designed to determine the minimum dose of D-tagatose required to affect HbA1c:

- D-tagatose at dosages of 2.5 g, 5.0 g, and 7.5 g TID for six months were well tolerated by this subject population.
- D-tagatose at 5.0 g TID was the minimal dose required to reduce HbA1c.
- D-tagatose at 7.5 g TID provided the greatest effect in most measured efficacy parameters.
- Future research might investigate the elevation of mean triglycerides with the 5.0 g D-tagatose dose group after three months of treatment. However, this elevation was transient and did not appear at the 6 month timepoint. This elevation was also only observed in the ITT population. If it were a drug effect, it would also likely appear in the EE population.

Acknowledgements

Credit

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Appendix C

Effect of BSN272 on Hyperlipidemia and Atherosclerosis in LDLr$^{-/-}$ Mice[1]

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Introduction[1]

The two objectives of this study were to (1) compare the effect of D-tagatose with the effect of a combination of D-tagatose and polydatin on serum triglycerides and cholesterol, and (2) compare the effect of these two treatments on the development of atherosclerosis in LDLr$^{-/-}$ mice.

In western countries, cardiovascular disease is the leading cause of mortality. While there are multiple factors that increase the risk of developing cardiovascular disease, there is evidence supporting a strong link between abnormal blood lipids (dyslipidemia) and increased risk for cardiovascular disease. Dyslipidemia is typically characterized by elevated levels of triglycerides and low-density lipoprotein (LDL) cholesterol and by low levels of high-density lipoprotein (HDL) cholesterol. Atherosclerotic lesions form from lipoproteins, macrophages, and lymphocytes in arterial blood vessels.[2] Blood cholesterol moves into damaged vessel endothelium layers. These modified lipids, changed by oxidation, cause macrophages and lymphocytes to enter the area to remove the LDLs. Over time, these components develop into plaque. Macrophages will display the lipoproteins on their cell surface and form foam cells initiating the plaque formation.

BSN272 is a combination drug therapy composed of a carbohydrate, D-tagatose, and polydatin, a glucoside derivative of resveratrol. 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.[3,4] Clinical studies have shown D-tagatose to be a potential anti-diabetic drug through its beneficial effects on postprandial hyperglycemia and hyperinsulinemia.[4] In addition to treating diabetes, D-tagatose may also be an effective treatment for obesity[4–6] and for reducing cardiovascular risks by increasing high-density lipoprotein (HDL) levels.[4] After over 10 years of animal and human studies, D-tagatose was classified as being "generally recognized as safe (GRAS)" by the FDA[7] and has been used since in food and beverage products.

Police *et al*.[8] found that the equivalent substitution of D-tagatose for sucrose as a dietary carbohydrate did not result in the same extent of obesity, hyperglycemia, hyperlipidemia, and atherosclerosis in LDLr$^{-/-}$ mice. Mice fed standard lab chow and mice fed D-tagatose chow exhibited similar energy intake, body weights and blood glucose and insulin concentrations, while sucrose-chow fed mice exhibited increased energy intake and became obese and hyperglycemic. Sucrose-fed mice had increased serum cholesterol, triglyceride concentrations and atherosclerosis compared to mice fed D-tagatose or a standard diet.

Polydatin is a natural substance that is a glucoside form of resveratrol. Evidence suggests that resveratrol, a naturally occurring polyphenol commonly found in a variety of plants and foods, most notably grapes, can produce a variety of beneficial effects, including the promotion of weight loss,[9] anti-oxidant properties[10] and cardioprotective,[11,12] anti-inflammatory and neuroprotective properties.[13] Recently, it was found that the concentration of polydatin in grapes is as much as seven times that of resveratrol[14,15] and is probably the most abundant form of resveratrol in nature.[16] Polydatin has a number of advantageous properties that increase its bioavailability compared to resveratrol, including a greater resistance to enzymatic oxidation.

Polydatin enters cells by an active transport mechanism using glucose carriers, unlike resveratrol which penetrates the cell passively.[14] A number of studies suggest that polydatin has biological properties similar to those of resveratrol.[17] Current evidence suggests polydatin may inhibit platelet accumulation, improve microcirculation, decrease lipid peroxidation, and reduced neutrophil-endothelial aggregation.[18] These proactive factors may limit the growth of plaque in arteries.

There is considerable interest in the use of trans-resveratrol and its derivatives, including polydatin, for the treatment of many human diseases.[19] 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.[20] 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,[14,21–23] cocoa,[24] peanuts and peanut butter,[25] pistachios[26] and almonds[27]. As a derivative of resveratrol, polydatin is believed to have many of the same beneficial effects, but also 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 in some ways 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.[10,28] These properties suggest that polydatin would have greater bioavailability than resveratrol.

Claims for health benefits of polydatin abound. Studies almost too numerous to count have presented evidence that polydatin has many positive effects including anti-inflammatory,[29,30] hepatoprotective,[31–34] anti-cancer,[35–38] neuroprotective,[29,39–41] and cardioprotective activities.[11,12,20,42,43] Pharmacological studies and clinical practice have demonstrated that polydatin also has protective effects against shock,[44–46] ischemia/reperfusion injury,[34,47] congestive heart failure,[48] endometriosis,[49] and prevention of fatty liver disease and insulin resistance,[50] and that it can regulate glucose and lipid metabolism.[51] Polydatin has recently participated in clinical trials for the treatment of hemorrhagic shock and irritable bowel syndrome.[30,52]

The way in which 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,[53,54] activation of protein kinase C,[18,55] suppression of NF-kappaB,[56] inhibition of the activation of renin-angiotensin-aldosterone system and decreasing the excretion of endothelin 1, TNF-α, and angiotensin II,[42] reduction of lipid peroxidation levels,[10,56] up regulation of the expression of hippocampal brain-derived neurotrophic factor,[41] enhanced insulin sensitivity in the liver as shown by improved insulin receptor substrate 2 expression levels and Akt phosphorylation,[51] decreasing the content of malonydialdehyde (MDA),[40] promoting the activities of total superoxide dismutase (T-SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) in plasma, increasing the content of glutathione (GSH) in myocardial tissue,[54] restoring decreased deacetylase sirtuin-1 activity and protein expression in liver tissue following severe shock[57] and activation of sirtuin,[58,59] and suppressing oxidative stress-induced lysosomal instability and mitochondrial injury by increasing the protein expression of SOD2.[57]

The treatment of dyslipidemia using polydatin has been suggested by a number of studies using animal models.[11,12,60] Polydatin given orally at 100 mg/kg lowered low-density lipoprotein (LDL) cholesterol by approximately 18% and serum triglycerides by 40% in rats consuming a standard chow containing a mixture of corn oil, 10% cholesterol, and 1% cholic acid.[60] Lower doses of trans-

polydatin (50 mg/kg body weight) were ineffective at preventing hyperlipidemia, however they were able to prevent the accumulation of cholesterol and triglycerides in the liver. In a study using Syrian golden hamsters, polydatin decreased total cholesterol levels and total triglyceride levels by 47% and 63%, respectively, in hamsters on a high fat, high cholesterol diet.[12] In a study using rabbits, polydatin lowered the serum levels of total cholesterol, triglycerides, and LDL.[11] The ratio of total cholesterol to HDL was reduced as well. In our laboratory, the combination of polydatin and D-tagatose has been shown to reduce cholesterol, triglycerides, and the extent of atherosclerosis in ApoE$^{-/-}$ mice.[61,62] The ApoE$^{-/-}$ mouse model is generally resistant to obesity, shows increased VLDL and LDL, and decreased HDL, and not particularly subject to developing insulin resistance.[63]

In the present study, we have examined the effect of BSN272, a combination of D-tagatose and polydatin, on blood lipids and atherosclerosis in LDLr$^{-/-}$ mice. In contrast to the ApoE$^{-/-}$ mouse model, in the LDLr$^{-/-}$ mouse model obesity, increased LDL, and insulin resistance are induced by a high fat diet. VLDL does not increase in the LDLr$^{-/-}$ mouse as it does in the ApoE$^{-/-}$ mouse. These differences might affect the development of atherosclerosis in this model, and lead to differences between the ApoE$^{-/-}$ and LDLr$^{-/-}$ mouse results.[1]

Methods[1]

Mice

Male 6-7 weeks old C57BL6-LDLr knockout (LDLr$^{-/-}$) mice (JAX Strain Name: B6.129S7-*Ldlr*^{tm1Her}/J) were used for this study. Mice were acclimated for 2 weeks prior to start of the study and were individually housed in solid bottom cages and kept on a standard light cycle: 12-hours light, 12-hours dark at 72 ± 8°F.

Treatment

This study was carried out at Covance Laboratories (Madison, WI). Animals were randomized by body weight into four groups (Illustration 1). Mice in group 1 (Control group, n=10) were dosed with water, while mice in group 2 (n=10) were dosed with 50% glucose + 50% fructose (see Table 1). The remaining mice were placed into groups 3 and 4 and randomly selected doses for animals 21-30 (group 3) and 31-40 (group 4) were forced to be uncorrelated by principal axis transformation. This orthogonalization of doses allowed the contribution to the reduction of lipids by polydatin and each sugar to be measured independently while still being in the presence of the other molecules.

For treatment groups 3 and 4, D-tagatose was added to ground feed (meal) each day. Groups 1 & 2 had ground TD.2014 (Teklad, Harlan Laboratories, Madison, WI) with no D-tagatose added. The dose in the feed of D-tagatose for groups 3 and 4 was increased by ~7.1% daily during the D-tagatose lead-in phase until the final maximum dose was reached. Individual animal feed bags were provided for each day of the lead-in phase of the study for all animals. Remaining feed was disposed of daily and the cages cleaned of the remaining crumbled feed. Duration of lead-in phase was 14 days. During the lead-in period all mice were handled daily to acclimate the animals to dosing by scuffing the animal to simulate gavage dosing. The study design is summarized in Illustration 1.

On Day 15 all animals were placed on TD.2014 for the remainder of the study. Mice were dosed by gavage based upon the most recent body weight, twice per week for 9 weeks. Table 1 shows the components in the solutions given to the mice by gavage. Each animal in groups 3 and 4 had a different formulation as shown in Table 2 “Glucose, Fructose, D-tagatose, and Polydatin (Piceid)

Doses for the Groups 2 - 22". Inside each dose group, the doses ranged from 0 to 0.853 g/kg/dose for the sugars and 0 – 0.150 g/kg/dose for polydatin. Dose volume was 10 mL/kg. Animals were weighed and food consumption was measured weekly. Blood samples were taken from the tail vein. Animals were not fasted prior to taking blood samples. On day 78 animals were anesthetized with isoflurane, bled by cardiac puncture, and the tissues removed.

Illustration 1[1]

Study Design

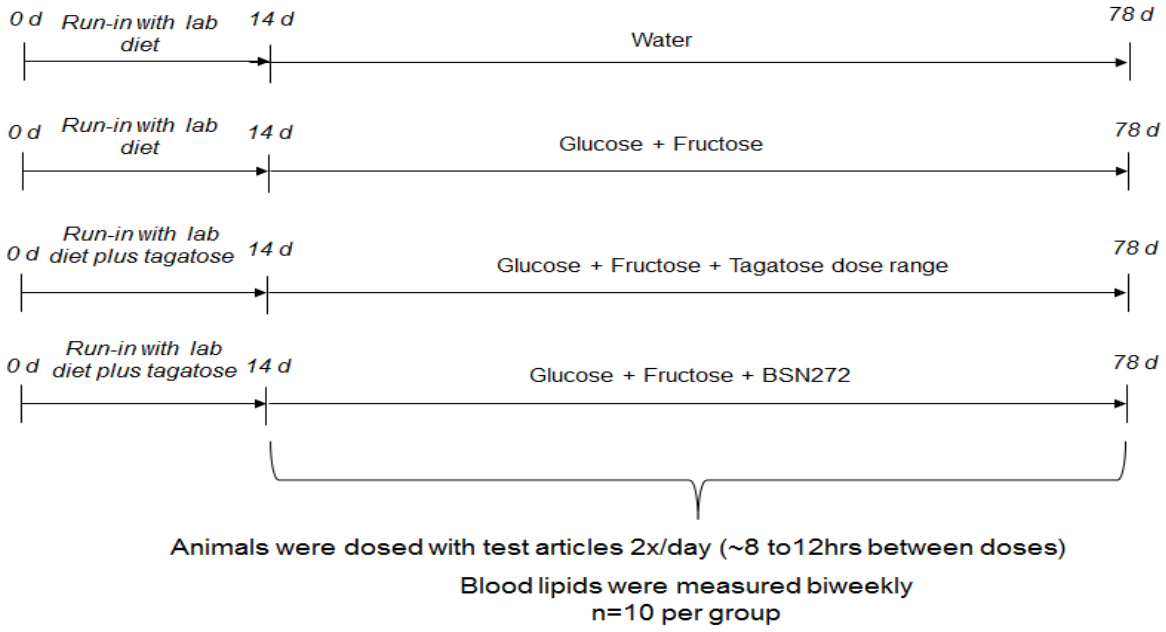


Table 1[1]

Compositions of Test Articles

Group	Test Article	Dose (g/kg/dose)	No. of animals	Animal Numbers
1	Water	0	10	1-10
2	Glucose (50%) + Fructose (50%)	See Table 4.2	10	11-20
3-12	Glucose + Fructose + D-tagatose	See Table 4.2	10	21-30
13-22	Glucose + Fructose + BSN272	See Table 4.2	10	31-40

Serum Lipids

Blood samples were obtained through tail cuts every two weeks throughout the experiment, and triglycerides, cholesterol, and free fatty acids were measured at Covance Laboratories using a Roche Hitachi 917 or Cobas 6000 analyzer using a photometric:enzymatic method. Animals were not fasted prior to bleeding, but were bled approximately 1 hour post dosing. On the final day of the study, mice were anesthetized with isoflurane and bleed by cardiac puncture. Body weights and food consumption were measured weekly.

At the end of the study mice were sacrificed via cardiac puncture. Aortic arches were harvested nine weeks after treatments began and the extent of atherosclerotic lesions was determined by false color imaging. Lesion area was measured as a fraction of the aortic arch area. The percent of atherosclerotic lesions in the BSN272 treated group was determined by dividing the mean

atherosclerotic lesion in the Glu/Fruc/BSN272 group by the mean atherosclerotic lesion in the Glu/Fruc group.

The amount of VLDLs, LDLs, and HDLs were determined by collecting serum nine weeks after the treatments began, resolving the lipoprotein complexes by FLPC, and quantifying the amount of cholesterol in each FPLC fraction using an enzymatic cholesterol assay. Samples for analysis were chosen from 5 mice in each of the glucose + fructose and glucose + fructose + BSN272 groups. The samples selected had total cholesterol values closest to the mean.

Table 2[1]

Glucose, Fructose, D-tagatose, and Polydatin (Piceid) Doses for the Groups 2 - 22

		Glucose	**Fructose**	**D-tagatose**	**Piceid**				
Max Dose g/kg/dose		0.85	0.85	0.85	0.15				
Treat-ment Group	Animal ID #	Glucose % of Max Dose	Fructose % of Max Dose	D-tag % of Max Dose	Piceid % of Max Dose	Glucose g/kg per dose	Fructose g/kg per dose	D-tag g/kg per dose	Piceid g/kg per dose
2	11	100	100			0.853	0.853		
2	12	100	100			0.853	0.853		
2	13	100	100			0.853	0.853		
2	14	100	100			0.853	0.853		
2	15	100	100			0.853	0.853		
2	16	100	100			0.853	0.853		
2	17	100	100			0.853	0.853		
2	18	100	100			0.853	0.853		
2	19	100	100			0.853	0.853		
2	20	100	100			0.853	0.853		
3	21	80.400	62.898	57.269		0.686	0.537	0.489	
4	22	51.045	50.295	26.541		0.435	0.429	0.226	
5	23	30.795	56.061	62.352		0.263	0.478	0.532	
6	24	29.005	73.871	24.031		0.247	0.630	0.205	
7	25	81.740	30.755	28.551		0.697	0.262	0.244	
8	26	84.530	53.531	51.408		0.721	0.457	0.439	
9	27	28.240	100.000	66.251		0.241	0.853	0.565	
10	28	47.517	59.586	64.954		0.405	0.508	0.554	
11	29	36.231	80.392	52.726		0.309	0.686	0.450	
12	30	63.580	64.693	99.000		0.542	0.552	0.844	
13	31	70.349	98.153	58.858	32.229	0.600	0.837	0.502	0.048
14	32	60.832	44.416	77.855	65.495	0.519	0.379	0.664	0.098
15	33	54.023	38.120	27.662	68.525	0.461	0.325	0.236	0.103
16	34	74.222	54.630	72.261	59.700	0.633	0.466	0.616	0.090
17	35	64.854	26.207	24.886	31.961	0.553	0.224	0.212	0.048
18	36	19.168	59.020	57.426	95.572	0.164	0.503	0.490	0.143
19	37	52.884	75.504	56.847	55.287	0.451	0.644	0.485	0.083
20	38	15.852	34.153	80.537	15.447	0.135	0.291	0.687	0.023
21	39	37.104	70.449	15.971	46.317	0.316	0.601	0.136	0.069
22	40	83.795	32.432	60.780	62.550	0.715	0.277	0.518	0.094

Table was obtained from Covance Protocol 8242054. Gavage formulation/dosing table for each animal in each group. The water group (negative control) is not shown. Doses in the Glucose/Fructose group (Treatment 2, positive control) are not orthogonalized. Doses in the Glucose/Fructose/Tagatose treatment group and the Glucose/Fructose/BSN272 treatment group are orthogonalized using principal axis transformation so multiple linear regression of treatment responses yields coefficients that do not change when one term (e.g., one drug) is added or dropped from the model. In MLR modeling, D-tagatose lowers triglycerides by -3.1 mg/dl per g/kg/dose of the sugar in the combination, while the polydatin lowers triglycerides by -372 mg/dl per g/kg/dose of the drug in the combination. D-tagatose lowers cholesterol by -3.9 mg/dl per g/kg/dose of the sugar in the combination, while the polydatin lowers cholesterol by -629 mg/dl per g/kg/dose of the drug in the combination. Note that the mass of the dose of D-tagatose is about an order of magnitude greater than polydatin in BSN272, so the net reductions of triglycerides and cholesterol due to tagatose in actual formulations would be about 10x greater. Paradoxically, in some animal models without D-tagatose, polydatin alone can raise serum triglycerides. In these animals adding D-tagatose causes polydatin to lower triglycerides.[1]

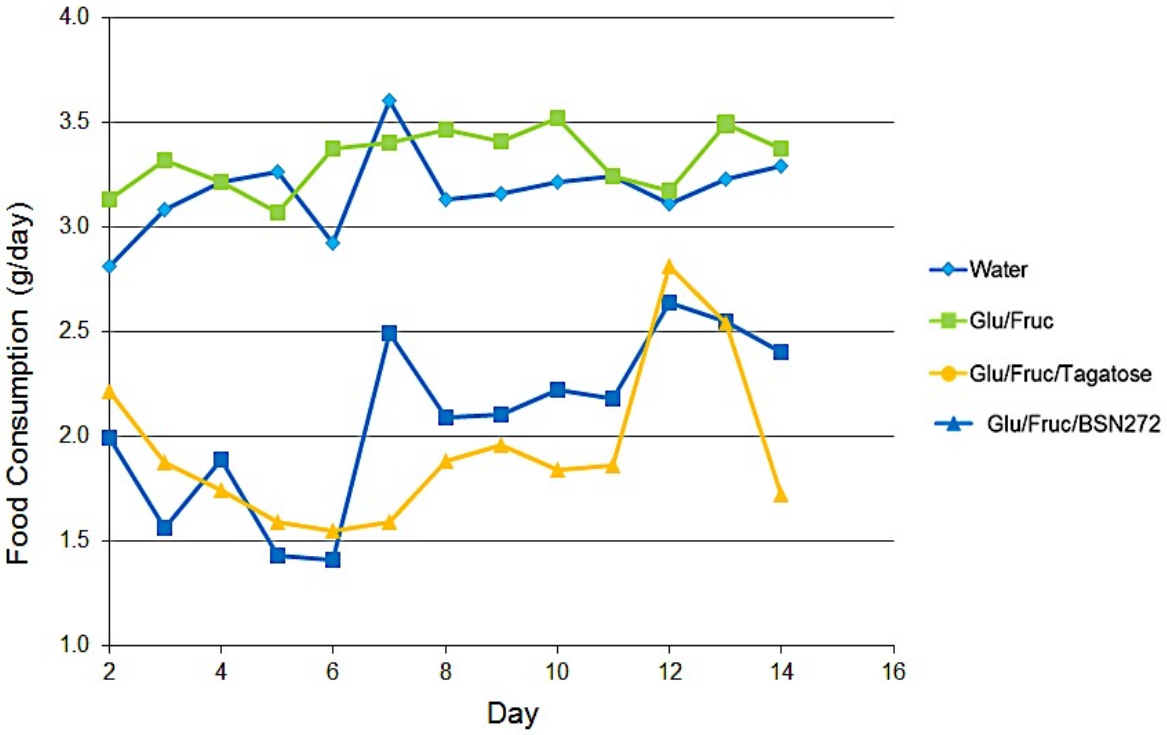
Results and Discussion[1]

Food Intake

There was no significant difference in amount of food eaten between mice in the different groups once treatments were started. There were differences in amount of food eaten during the two-week D-tagatose lead-in (Illustration 2). Mice being fed the D-tagatose during the lead-in phase ate less (1.9 ± 0.10 g for Groups 3-13 and 2.1 ± 0.11 g for Groups 13-22) than mice in Group 1 (3.2 ± 0.052 g) or 2 (3.3 ± 0.04 g). This was somewhat expected as studies in humans have found D-tagatose produced a feeling of satiety.[4,64–66] Once mice were place on the standard chow and gavage treatments began, food consumption was the same for all three groups (Illustration 3).

Illustration 2[1]

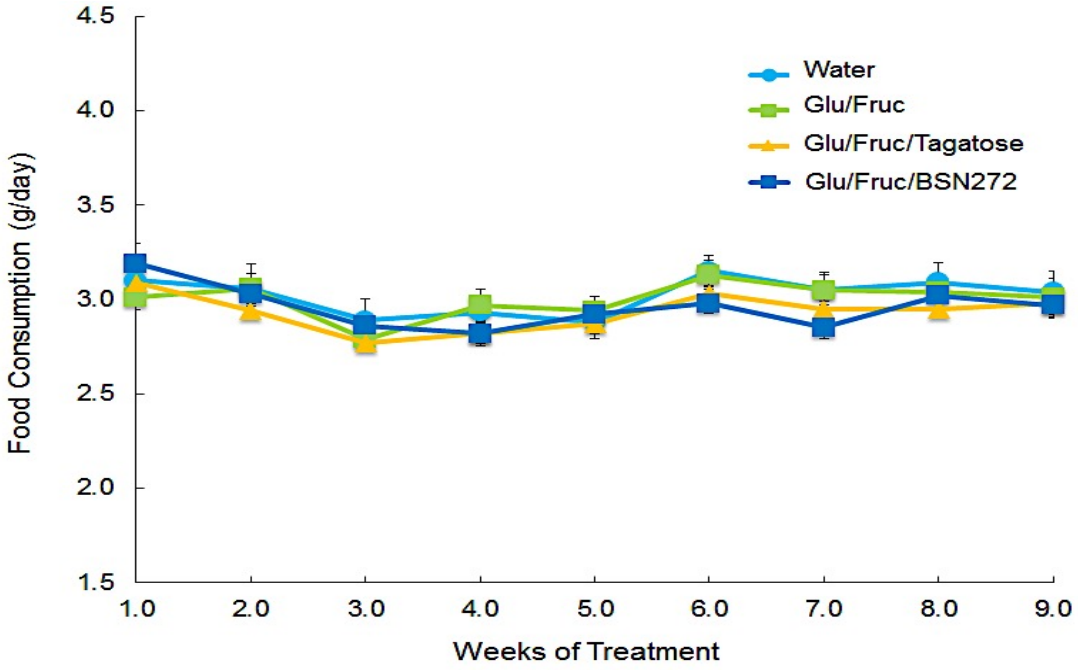
Food Consumption During D-tagatose Run-In



Food consumption during the 14-day D-tagatose lead-in period during which animals in Groups 3 and 4 were given increasing amounts of D-tagatose each day to acclimate them to the sugar.[1]

Illustration 3[1]

Food Consumption During Treatment



Food intake measured by weight of food eaten per day was the same for all four groups after the 14-day D-tagatose lead-in period.[1]

Body Weights

Mice in the D-tagatose and BSN272 groups weighed slightly less at the start of the study before any treatment began than mice in the water or Glu/Fruc groups. This slight difference was maintained throughout the course of the experiment. Even though mice in the groups receiving the D-tagatose during the 2-week lead-in phase ate less (see Illustration 2), their weight gain during the lead-in phase was no different than mice not receiving the D-tagatose that ate more. There were no significant differences in body weights of the mice in the four groups during the course of the study (Illustration 4). The rate of weight gain was similar for all mice during the course of the study.

Tagatose and BSN272 Reduce Serum Lipids in LDLR$^{-/-}$ Mice

Cholesterol

Day 78, end of study result. Glucose/Fructose raised total serum cholesterol in LDLr$^{-/-}$ mice compared to control mice. Treatment with D-tagatose or BSN272 prevented the increase due to the glucose/fructose (Illustration 5). End point mean cholesterol was 322 ± 18 mg/dl for the control group, 378 ± 18 mg/dl for the Glucose/Fructose group, 309 ± 27 mg/dl for Glucose/Fructose/Tagatose group, and 305 ± 16 mg/dl for the Glucose/Fructose/BSN272 group (Illustration 5).

Illustration 4[1]

Body Weights of Mice Throughout Study

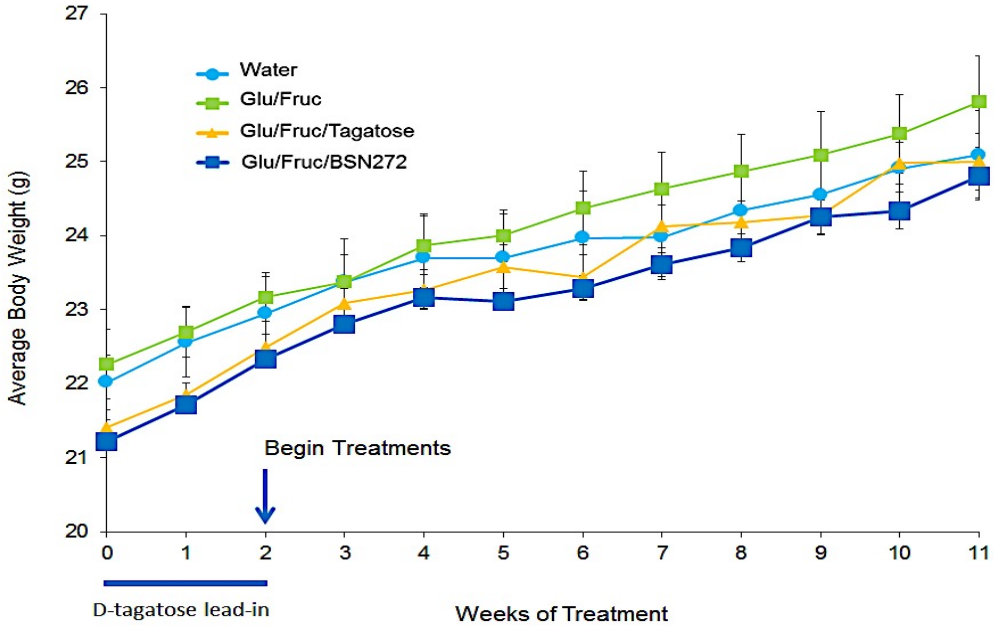
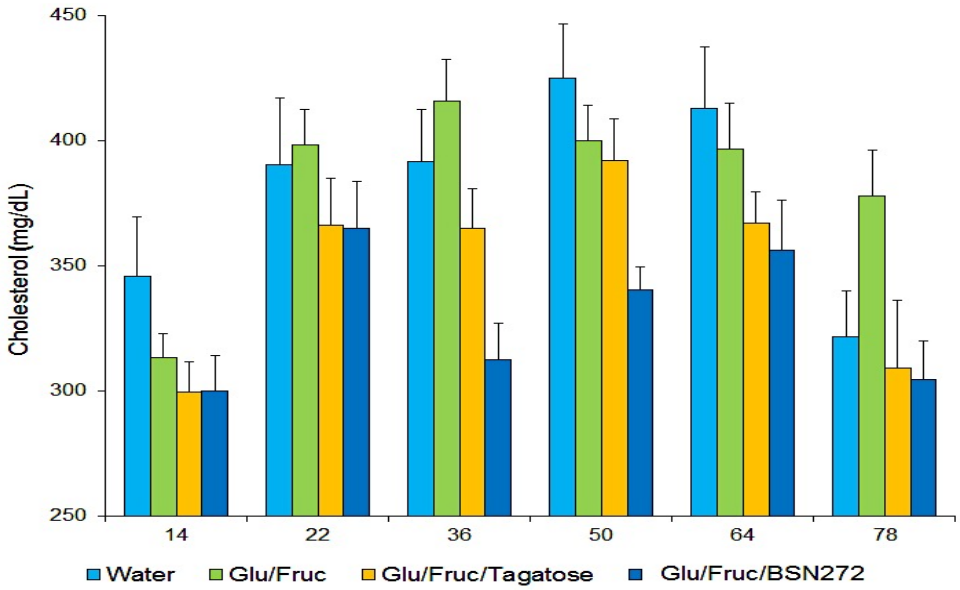


Illustration 5[1]

Total Serum Cholesterol



The total serum cholesterol of fed mice treated with D-tagatose or BSN272 is always lower than for mice treated with glucose/fructose or even water.[1]

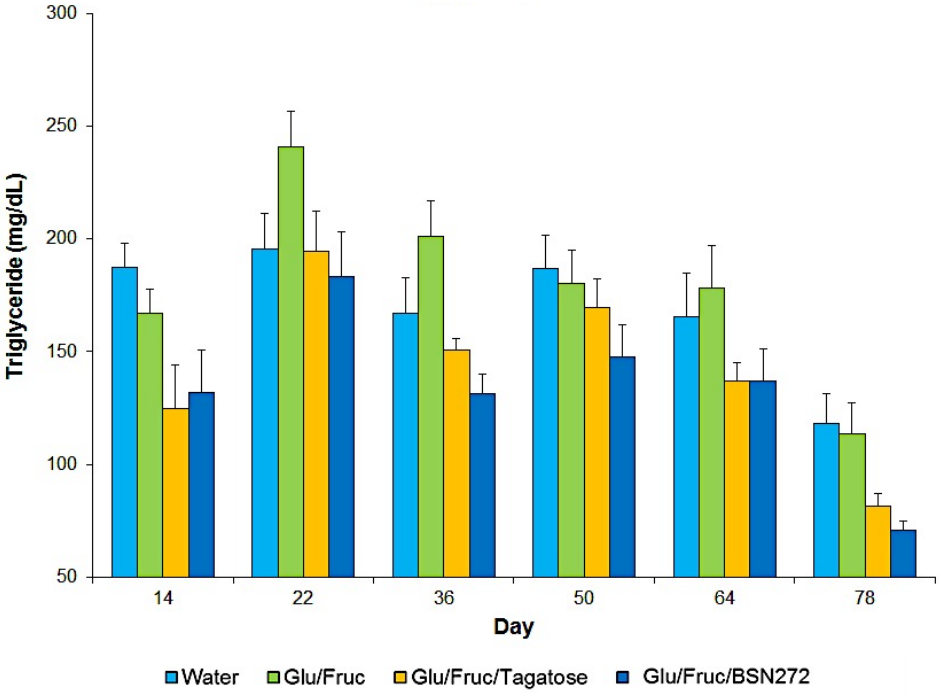
Triglycerides

Day 78, end of study result. Glucose/Fructose did not change serum triglyceride levels compared to control mice (118 ± 14 mg/dl for Glucose/Fructose mice compared to 114 ± 13 mg/dl for control mice). However, treatment with D-tagatose or BSN272 reduced serum triglyceride levels (81 ± 6

mg/dl and 71 ± 4 mg/dl, respectively), with the BSN272 having the lowest study end point triglyceride level (Figure 5.6).

Illustration 6[1]

Serum Triglycerides



The total serum triglycerides of mice treated with D-tagatose or BSN272 is always lower than for mice treated with glucose/fructose or even water.[1]

Free Fatty Acids

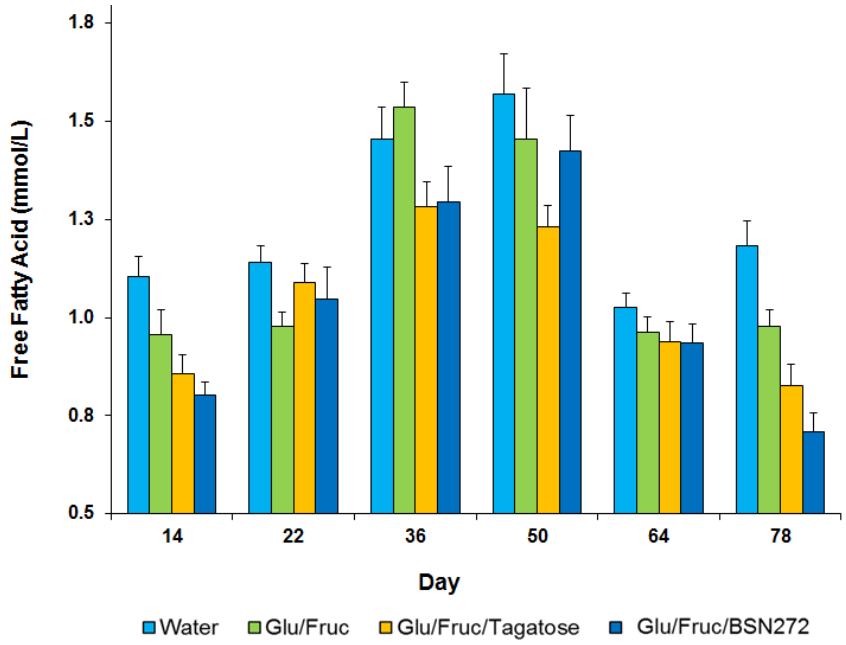
Free fatty acid levels in all groups on Day 14 look approximately the same as their respective levels on Day 78 when the study ended (Illustration 7). In the middle of the study (days 36 and 50) values for all groups go up by about 50%. On day 14 the D-tagatose and BSN272 groups have significantly lower fatty acid levels than the glucose/fructose or water control groups, which could be due to the D-tagatose given during the lead-in phase. Fatty acids drop considerably for all four groups from Day 50 to 64, and then go back up in the group on water (control) and drop in the BSN272 group. The drop in all groups makes it difficult to determine if any change is treatment related. There is no statistically significant difference between the D-tagatose and BSN272 groups at any time point.

BSN272 Reduces VLDL and LDL in LDLR$^{-/-}$ Mice

The amount of VLDLs, LDLs, and HDLs were determined by collecting serum nine weeks after the treatments began, resolving the lipoprotein complexes by FLPC, and quantifying the amount of cholesterol in each FPLC fraction using an enzymatic cholesterol assay. A 25% trimmed mean FPLC chromatogram was calculated using the total cholesterol values from the enzymatic cholesterol assay. BSN272 reduced VLDL and LDL by 35% and 17%, respectively ($p<0.05$). HDLs were not significantly altered by treatment (Illustration 8).

Illustration 7[1]

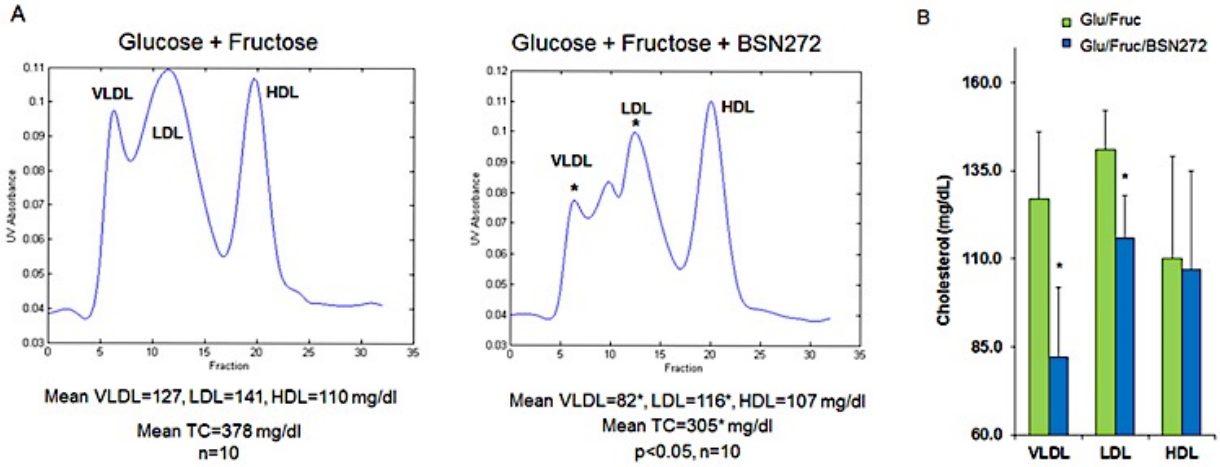
Free Fatty Acids



There is no statistically significant difference between the D-tagatose and BSN272 respective groups at days 14 and 78.[1]

Illustration 8[1]

VLDL, LDL, and HDL Measurements



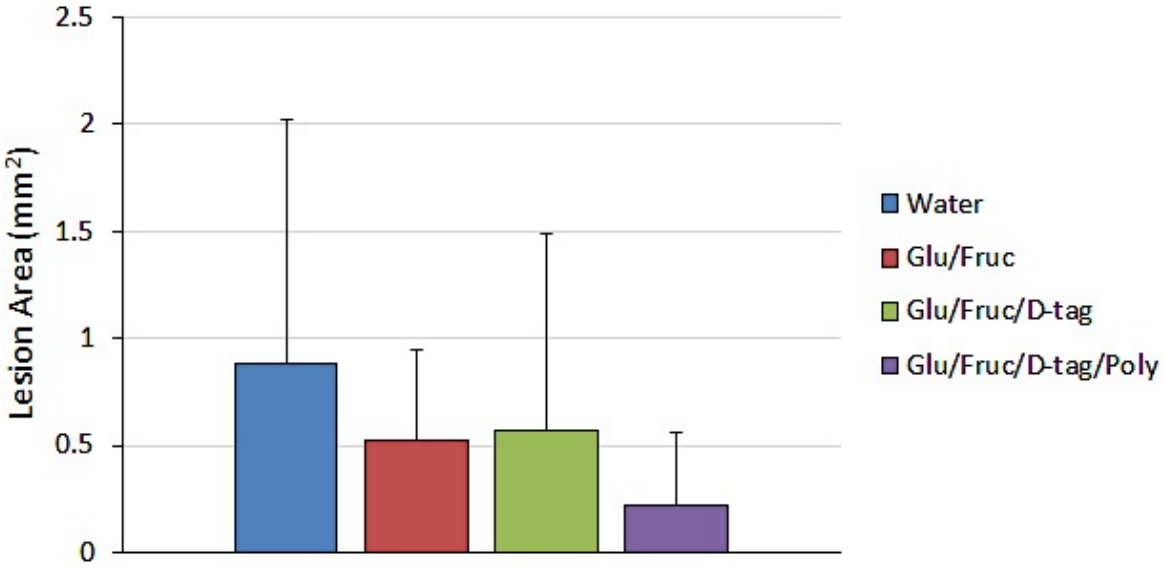
(A) Trimmed mean VLDL, LDL and HDL FPLC chromatograms at 9 Weeks. (B) Treatment with BSN272 significantly reduces VLDL and LDL while leaving HDL essentially unchanged.[1]

BSN272 Reduces Atherosclerotic Lesions in LDLr$^{-/-}$ Mice

Aortic arches were harvested 9 weeks after treatments began and the amount of atherosclerotic lesion was determined by false color imaging (Illustration 9). Lesion area was measured as a fraction of the aortic arch area. The percent of atherosclerotic lesions in the BSN272 treated group was determined by dividing the mean atherosclerotic lesion area in the Glu/Fruc/BSN272 group by the mean atherosclerotic lesion area in the Glu/Fruc group. BSN272 in the diet reduced atherosclerotic lesions to less than one-half of their original level.

Illustration 9[1]

Aortic Arch Lesion Area



Effects of combination of D-tagatose and BSN272 on atherosclerosis in LDLr-deficient mice. Atherosclerotic lesion area was determined by false color imaging.[1]

Factor Analysis Results for the LDLr$^{-/-}$ Mice

The use of oral gavage doses orthogonalized by transformation to principal axes permits the efficacy of each molecule (D-tagatose and trans-polydatin) to be calculated in the presence of the other. The BSN272 combination is the most effective of the treatments for lowering triglycerides.

- Both glucose and fructose raise triglycerides.
- D-tagatose lowers triglycerides by -3.1 mg/dl per g/kg/dose of the sugar in the combination of BSN272, while the polydatin lowers triglycerides by -372 mg/dl per g/kg/dose of the drug in the combination.

The polydatin/tagatose combination (BSN272) is the most effective of the treatments for lowering cholesterol.

- Both glucose and fructose raise cholesterol.
- D-tagatose lowers total cholesterol by -3.9 mg/dl per g/kg/dose of the sugar in the combination, while the polydatin lowers total cholesterol by -629 mg/dl per g/kg/dose of the drug in the combination.

Paradoxically, in some animal models, D-tagatose and polydatin alone can raise serum triglycerides. Polydatin administered alone in the Syrian Golden hamster on Western diet increases serum triglycerides. D-tagatose administered alone in the Syrian Golden hamster on Western diet increases serum triglycerides. Polydatin co-administered with D-tagatose (BSN272) in the Syrian Golden hamster on Western diet decreases serum triglycerides (unpublished study). Unlike the LDLr$^{-/-}$ mouse, the hamster has cholesterylester transfer protein (CETP), similar to humans. CETP transports cholesteryl esters and triglycerides between the lipoproteins. CETP can pick up triglycerides from very-low-density (VLDL) or low-density lipoproteins (LDL) and swap them for cholesteryl esters from high-density lipoproteins (HDL), and vice versa.[67]

BSN272 Results Summary

- Serum triglycerides (TG) were reduced by almost one-half. However, there was also a reduction in TG in mouse on water treatment, making it difficult to conclude that TG reduction is treatment related in the LDLr-/- mouse.
- Reduction on VLDL cholesterol was the next largest, followed by the reduction in LDL cholesterol.
- Reduction in TG, VLDL, and LDL may explain the reduction in atherosclerotic lesion area in the aortic arch.

Conclusions[1]

This study was designed to compare the effects of D-tagatose alone and in BSN272 on the levels of cholesterol and triglycerides and on preventing atherosclerosis in $LDLr^{-/-}$ mice. $LDLr^{-/-}$ mice maintained on a high-fat diet provide a model of hypercholesterolemia with somewhat elevated plasma cholesterol.[68] In addition, Zadelaar et al. found that the lipoprotein profile in $LDLr^{-/-}$ mice closely mimics that of humans, with the cholesterol mainly tied up in the LDL fraction.[69] $LDLr^{-/-}$ mice were used to study the effects of BSN272 versus D-tagatose alone on lipid levels in these mice, and to compare the $LDLr^{-/-}$ model with results provided by the $ApoE^{-/-}$ model used in other published studies.

A diet that was supplemented with Glucose/Fructose and D-tagatose or BSN272 produced no significant change in the food intake or body weight of $LDLr^{-/-}$ mice. However, free fatty acids and lipids, including triglycerides and total cholesterol, significantly decreased in mice given BSN272. Serum triglycerides (TG) were cut almost in half. LDL and VLDL, but not HDL, levels were also decreased. Not surprisingly, aortic atherosclerotic lesions were reduced by 57%, as BSN272 reduced the amount of lipids moving through the blood.

Castelli found that cardiac events peak in individuals with LDL levels of 150 mg/dl.[70] BSN272's ability to suppress LDL formation could significantly deter future cardiac impairment. Castelli postulated that small dense VLDL particles settle in vessels and participate in forming plaque, while "fluffy" VLDLs simply travel back to the liver for excretion.[70] These dense VLDLs are likely to become circulating LDLs at some point. As such, suppression of VLDL formation could reduce arterial plaque formation. It is thought that at a triglyceride level of 150 mg/dl, only small dense pattern B LDLs are being formed as opposed to fluffy "likely to be excreted" LDLs. BSN272 reduced VLDL by 35%.

It is well documented that elevated levels of LDLs can contribute to lipoprotein retention, and higher levels of anti-inflammatory markers.[71] Presently, statins are often prescribed as lipid lowering therapies, however, in a study of over 4000 patients, only 40% of patients being treated with a statin drug regimen were able to meet target LDL-C levels.[72] Additionally, statins are mostly ineffective in reducing triglycerides. In a study of $LDLr^{-/-}$ mice fed a high cholesterol (1%) diet described by Wang *et al*, simvastatin dosed at 300 mg/kg decreased serum LDL cholesterol levels from 917± 80 mg/dl in control mice to 322 ± 27 mg/dl in simvastatin treated mice and reduced aortic lesion area by fifteen percent. However, the treatment had no effect upon triglyceride levels. In the same study, ApoE mice fed the same diet and given the same dosage of statin, had an increase of 27% in serum cholesterol.[73] Apolipoprotein E, which transports cholesterol into cells, is believed to ferry cholesterol into hepatocytes for metabolic clearance. Additionally, statins, including simvastatin, work by inhibiting HMG-CoA reductase, an enzyme responsible for catalyzing cholesterol production. In other studies with LDLr deficient mice, LDL and total plasma cholesterol were significantly lowered with atorvastatin (-41 and -27%), lovastatin (-27 and -21%)

and simvastatin (-22 and -15%), but not with control (+8 and +11%), and there was no significant change in triglycerides,[74] whereas in this study BSN272 produced a 17% decrease in LDL levels.

BSN272 differs from Lovaza and other omega-3-acid ethyl esters in that it not only reduces triglycerides it also reduces LDLs. Triglyceride levels have been found to be particularly important to women as the Farmingham Study found women with triglyceride levels greater than 150 mg/dl and HDL cholesterol levels below 50 mg/dl have one the highest rates of coronary heart disease (CHD).[70] Various studies have found that Omacor does significantly reduce mean triglyceride concentrations, including the Harris study (1997) which reported a 45% reduction in triglycerides, increased HDL cholesterol by 13% and LDL cholesterol by 31%, dosed at 3.4 g eicosapentaenoic acid (EPA) and docosapentaenoic acid (DHA) and 18 mg vitamin E per day.[75] Additionally, the Pownall (1999) study reported a 38.9% decrease from baseline fasting triglyceride levels and an increase in HDLs of 5.9% and an increase in LDLs of 16.7%.[76] EPA and DHA inhibit acyl-COA, resulting in decreased triglyceride synthesis, while increasing fatty acid metabolism, and increasing lipase production which results in increased triglyceride binding.[77]

Foam cell formation is one of the early steps in the process of atherosclerosis.[78] Free metal ions can help to oxidize the LDL and convert it into a form that can be taken up by macrophages. BSN272 seems to prevent LDL oxidation and may prevent oxidation caused by macrophages at the atherosclerotic site.

Although glucose and fructose had modest effects on serum lipids and body weight over the course of the study, the levels of serum cholesterol and triglycerides in the groups receiving glucose, fructose, and D-tagatose, and glucose, fructose, D-tagatose, and BSN272 were consistently equal to or less than the levels in the groups receiving either water or glucose and fructose. Furthermore, the combination of D-tagatose and polydatin appeared to be more potent than D-tagatose alone in reducing serum cholesterol and triglycerides over the course of the experiment. D-tagatose and polydatin also prevented the increase in serum cholesterol induced by glucose and fructose, and was capable of reducing atherosclerosis in LDLr-deficient mice.

Importantly, the sera collected on days 14, 22, 36, 50, and 64 were from fed (not fasted) mice, making it difficult to clearly define a role for D-tagatose and polydatin on the metabolism of endogenous lipids. However, considering that the levels of serum triglycerides and cholesterol in the mice treated with either glucose, fructose, and D-tagatose or glucose, fructose, D-tagatose, and BSN272 were always less than or equal to those in mice receiving either water or mixtures of glucose and fructose and that blood samples were collected one hour after gavaging, these results suggest that supplementing high carbohydrate meals with D-tagatose or combinations of D-tagatose and BSN272 may be effective at reducing postprandial carbohydrate-induced hyperlipidemia and lowering serum cholesterol and triglycerides over time.

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Competing Interests:

Dr. Lodder was President of Biospherics at the time these data were collected.

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Appendix D

4-Week Toxicity and Toxicokinetic Oral Gavage Study with Polydatin in Rats[1]

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Introduction[1]

There is considerable interest in the use of polydatin for the treatment of a variety of human diseases.[3] Polydatin ((PD) also known as trans-polydatin, piceid, and 3,4',5-trihydroxystilbene-3-β-mono-D-glucoside) is a glucoside derivative of resveratrol. It is purified from the root of Polygonum cuspidatum but can also be found in wines and grapes,[4–7] cocoa,[8] peanuts and peanut butter,[9] pistachios[10] and almonds.[11] 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.[12] Polydatin is found to be the major component of these extracts. As a derivative of resveratrol, it 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 resistant to enzymatic breakdown than resveratrol.[13] It is also actively taken up by cells via glucose carriers in the cell membrane instead of being passively transported like resveratrol.[14,15] These properties suggest that polydatin may have greater bioavailability than resveratrol.

Studies have presented evidence that polydatin has many positive health effects. These effects include anti-inflammatory,[16,17] hepatoprotective,[18–21] anti-cancer,[22–25] neuroprotective[16,26–28] and cardioprotective activities.[12,29–32] Additional studies demonstrated that polydatin also has protective effects against shock,[33–35] ischemia/reperfusion injury,[36,21] congestive heart failure,[37] endometriosis,[38] prevents fatty liver disease and insulin resistance,[39] and that it can regulate glucose and lipid metabolism.[40] Polydatin has been studied in clinical trials for the treatment of hemorrhagic shock and irritable bowel syndrome.[17,41]

The use of polydatin as a potential therapy for dyslipidemia has been suggested by studies using animal models. Arichi *et al.* discovered that orally administered polydatin (100 mg/kg body weight) significantly lowered serum triglycerides by 40% and low-density lipoprotein (LDL)-derived cholesterol by approximately 18%. This study was performed 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, which may suggest that lower doses will also be effective but to a much lesser extent.[42] In a study using Syrian golden hamsters, polydatin decreased total cholesterol levels and total triglyceride levels by 47% and 63%, respectively, when compared to standard diet.[30] In another study using rabbits, the administration of polydatin decreased serum total cholesterol, triglycerides, and LDL.[29] The ratio of total cholesterol to HDL was also reduced.

Insulin is a major component of metabolic regulation through activation of the Akt and other metabolic pathways.[43] Hao *et al.* recently found in diabetic rats that polydatin activated the Akt signaling pathway, possibly by phosphorylation of the insulin receptor substrate (IRS), thus reducing blood glucose levels.[40] Polydatin may also decrease the expression of intercellular adhesion molecule 1 (ICAM-1) which could reduce white blood cell adhesion thought to be active

in early atherosclerotic development.[13] Polydatin is also thought to provide protection from cell damaging oxidative peroxidation[14,44] and inhibit oxidation of LDL particles, which may also play a role in atherosclerosis.[12]

Both human and animal studies suggest that orally administered polydatin is absorbed in minutes, metabolized in minutes to hours, and eliminated in 24 hr.[45–47] Metabolites of trans-polydatin include glucuronidated and/or sulfonated trans-polydatin, trans-resveratrol, and glucuronidated and/or sulfonated trans-resveratrol.

This study determined the toxicity and toxicokinetics of polydatin when administered via oral gavage to Sprague Dawley rats daily for 4 weeks.

Methods[1]

Regulatory Guidelines

The study was conducted according to Good Laboratory Practice (GLP) and based on the principles of the Food and Drug Administration Center for Drug Evaluation and Research (CDER)/International Conference on Harmonisation (ICH) Harmonised Tripartite Guidelines ICH-M3, Nonclinical Safety Studies for the conduct of Human Clinical Trials for Pharmaceuticals (CDER, July 1997) and S3A, Toxicokinetics: The Assessment of Systemic Exposure in Toxicity Studies (CDER, March 1995).

Animals

Male and female Crl:CD(SD) rats were received from Charles River Laboratories, Portage, Michigan. At initiation of dosing, the animals were 6 to 7 weeks old, and their body weights ranged from 238 to 303 g for males and 143 to 238 g for females. Animals were randomized to the study groups using a computerized procedure designed to achieve body weight balance with respect to subgroup assignment, within a 5% probability of homogeneity of variance. Following randomization, each study animal was assigned a unique number by means of an implantable microchip identification device and/or cage card. The oral route of administration was selected because it is the intended route of administration in humans.

Male and female rats were housed individually in stainless steel cages in the following conditions: temperature range of 20 to 26°C, relative humidity range of 30 to 70%, 10 or greater air changes/hour, and a 12-hour light/12-hour dark cycle. The light/dark cycle was interrupted for study-related activities.

Diets and Test Materials

Animals were offered Certified Rodent Diet #2016C (Harlan Laboratories, Inc.) ad libitum unless fasted for study procedures. Water was provided ad libitum.

Experimental Design

Testing was performed by Covance Laboratories (Madison, Wisconsin). The test article, polydatin, was supplied by Biotivia (purity 98.36% by HPLC, Lot. No. BIPL110714) and was stored at 2 to 8°C. The vehicle control article was reverse osmosis water. Test article formulations were prepared daily on Days 1 through 5 of the dosing phase and once weekly from day 6 through the remainder of the dosing phase. Formulations prepared prior to the day of dosing were stored in a refrigerator, set to maintain 2 to 8°C, and protected from light until removed for dosing. Formulations prepared on the day of dosing were stirred continuously using a magnetic stir bar

and stir plate and stored protected from light at room temperature. Formulations were kept at approximate room temperature no longer than 8 hours.

Animals were weighed 3 days prior to receiving their first dose, on the day they received their first dose, and weekly thereafter. All animals were checked twice daily (a.m. and p.m.) for mortality, abnormalities, and signs of pain or distress. At a minimum, clinical observations made from cage side were recorded daily during the dosing phase. Detailed clinical observations were recorded on toxicity animals once during the predose phase, prior to dosing on Day 1, and weekly (based on Day 1) throughout the dosing phase, and the termination date. Food consumption was measured for the toxicity groups. Ophthalmic evaluations were performed once during prephase and on Day 29 of the dosing phase by a qualified veterinarian.

Polydatin was administered by oral gavage once daily for 29 days (dosing phase) at a dose volume of 10 mL/kg. according to Table 1 - Study Groups. Doses were stirred for at least one hour prior to and during the dosing. The dose levels selected were based on available data from the literature of toxicity of resveratrol[48] in addition to studies evaluating efficacy of polydatin.[49–51] Statistical power analysis suggests 30 mg/kg is the minimum dose capable of causing a statistically significant reduction in cholesterol and triglycerides based on results in the LDLr$^{-/-}$ mouse.[52] The dose level of 300 mg/kg is an order of magnitude larger and is the dose for which there are published kinetic data in the literature.[46,53] The upper limit of 3000 mg/kg is another order of magnitude larger and a level at which some effects on the kidney have been reported.[54]

Table 1[1]
Study Groups

Group	Subgroup	No. of Animals		Dose Level (mg/kg/day)	Dose Concentration (mg/mL)
		Male	Female		
1 (Control)[a]	1 (Toxicity)	10	10	0	
2 (Low)	1 (Toxicity)	10	10	300	30
	2 (Toxicokinetic)	9	9	300	30
3 (Mid)	1 (Toxicity)	10	10	600	60
4 (Mid-High)	1 (Toxicity)	10	10	1200	120
5 (High)	1 (Toxicity)	10	10	3000	300
[a] Group 1 received vehicle control article only.					

After 29 days of dosing, all surviving toxicity animals were sacrificed and necropsied. Terminal body weights were recorded. Animals were anesthetized with sodium pentobarbital and exsanguinated. An examination of the external features of the carcass, external body orifices, abdominal, thoracic, and cranial cavities, organs, and tissues were performed. A pathologist was available for consultation.

Organ weights, including adrenal (2), brain, epididymis (2), heart, kidney (2), liver, lung, ovary (2) pituitary gland, prostate, salivary gland, mandibular (2), seminal vesicle, spleen, testis (2), thymus, thyroid (2 lobes) with parathyroid, and uterus, were recorded for toxicity animals. Paired organs were weighed together. Adrenal (2), aorta, brain, cecum, cervix, colon, duodenum, epididymis

(2), esophagus, eye (2), a femur with bone marrow (articular surface of the distal end), Harderian gland, heart, ileum, muscle (biceps femoris), optic nerve (2), ovary (2), pancreas, pituitary gland, prostate, rectum, salivary gland [mandibular (2)], sciatic nerve, seminal vesicle, skin/subcutis, spinal cord (cervical, thoracic, and lumbar), spleen, sternum with bone marrow, stomach, jejunum, kidney (2), lesions, liver, lung with large bronchi, lymph node (mandibular), lymph node (mesenteric), mammary gland (females), testis (2), thymus, thyroid (2 lobes) with parathyroid, tongue, trachea, urinary bladder, uterus, and vaginal tissues (when present) from each toxicity animal were preserved in 10% neutral buffered formalin, with the exception of the eyes, Harderian gland, optic nerves, and testes, which were collected in modified Davidson's fixative and stored in 10% neutral-buffered formalin.

For histopathology, tissues were embedded in paraffin, processed to slide, and stained with hematoxylin and eosin. All preserved tissues (see above) were examined for histopathologic irregularities from toxicity animals in the control and high-dose groups and kidney, liver, and macroscopic lesions from all animals in the low-, mid-, and mid-high dose groups were examined microscopically by a veterinary pathologist. In addition, based on microscopic findings in the control and high dose groups, the urinary bladders from 2 females in Group 3, Subgroup1 and the spleen from males in the low-, mid-, and mid-high dose groups were examined microscopically by the same veterinary pathologist. Additionally, any macroscopic lesions or suspected target organs of note at the high dose range, were examined.

Blood samples were collected from toxicity animals (see Table 1) for hematology, clinical chemistry, and coagulation via a jugular vein from animals fasted overnight. Samples were collected on the day of scheduled sacrifice. Sodium citrate and potassium EDTA were used as anticoagulants for coagulation and hematology tests, respectively. Samples for clinical chemistry were collected without anticoagulant. Urine samples for urinalysis were collected chilled on wet ice during the overnight period from fasted toxicity animals. Samples were collected on the day of scheduled termination.

Blood samples (approximately 0.25 mL) were collected prior to dosing via a jugular vein from toxicokinetic animals (Group 2, Subgroup 2, nine animals/sex, see Table 1) given 300 mg/kg/day predose (Day 25 only). Nine animals per sex were used with blood samples collected from three animals/sex/time point. Blood samples were collected from three animals/sex/timepoint on Days 1 and 25 at the following time points: predose (Day 25 only) and at approximately 0.167, 0.333, 0.5, 1, 2, 4, 8, 12, and 24 hours postdose. Samples were collected in tubes containing potassium EDTA and maintained on chilled cryoracks until centrifugation. Samples were centrifuged within 1 hour of collection, and plasma was harvested. Following centrifugation, samples were processed under yellow light. Plasma samples were stored in a freezer, set to maintain -60 to -80°C, until analyzed. Plasma analysis for trans-polydatin (also known as polydatin) and trans-resveratrol was performed by Covance-Madison using a method (Method SPXRPP) previously validated under Covance Study No. 8251389. Incurred sample reproducibility was conducted in accordance with Covance standard operating procedures.

Toxicokinetic analysis included (when appropriate), but was not limited to, maximum observed concentration (Cmax), time to peak concentration (Tmax), and area under the concentration-time curve (AUC). Area under the concentration-time curve from hour 0 to infinity for Day 1 calculated as follows: $AUC_{0-\infty} = AUC_{0-t} + C_t/\lambda_z$. Where C_t is the last measurable concentration and λ_z is the elimination rate constant estimated using log-linear regression during the terminal elimination phase. The number of points used in λ_z calculation was determined by visual inspection of the

data describing the terminal phase. At least the last three time points with measurable values were used in λ_z calculation.

Statistical Analysis

Data for each sex were analyzed separately; only data collected on or after the first day of dosing were analyzed statistically. Only data from toxicity animals (Subgroup 1) were evaluated. Analysis of variance (ANOVA)[55] and pairwise comparisons were used to analyze the following; absolute body weight, body weight change, quantitative food consumption, continuous clinical pathology values, terminal body weight, absolute organ weight, organ to body weight percentage, and organ to brain weight percentage.

Levene's test[56,57] was done to test for equality of variances between groups. Where Levene's test was significant ($p < 0.05$), a rank transformation (to stabilize the variances) was applied before the ANOVA was conducted (note: Levene's test was not applied to the rank-transformed data). Where Levene's test was not significant ($p > 0.05$), ANOVA was conducted.

One-way ANOVA was used (if applicable) to analyze continuous clinical pathology values, absolute organ weight data, food consumption, and body weight data. If the group effect of the ANOVA was significant ($p < 0.05$), Dunnett's t-test[58,59] was used for pairwise comparisons between each treated and control groups. Group comparisons (Groups 2 through 5 versus Group 1) were evaluated at the 5.0%, two-tailed probability level.

Results[1]

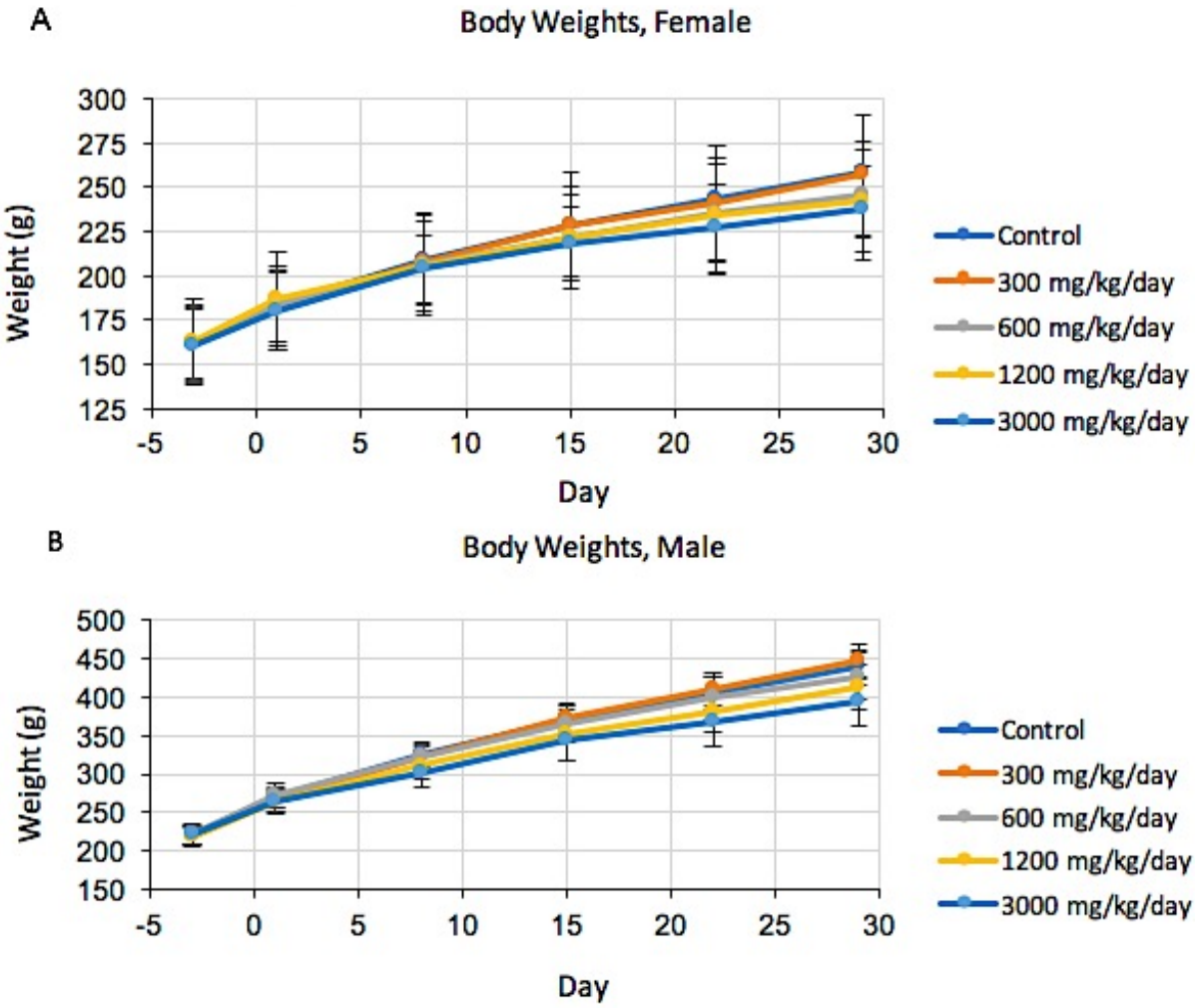
Body Weights

Animals given 600, 1200, or 3000 mg polydatin/kg/day showed lower mean body weights which were dose dependent (Figure 1). Decreases were of small magnitude (≤10% compared with control) and correlated with lower body weight gain in these animals (Figure 2). Statistically significant decreases included lower mean body weight for all 4 weeks of the dosing phase for males given 3000 mg/kg/day. While the decreases were not always statistically significant, dose dependency and a correlating decrease in food consumption suggested decreases in body weight and body weight gain were polydatin related. Due to the absence of relevant clinical observations and small magnitudes of the changes, decreases in body weight and body weight gain were not considered adverse. In addition, polydatin is a prodrug of resveratrol, and resveratrol is a caloric restriction mimetic.[60,61] For this reason, a dose-dependent decrease in body mass is not surprising (See Table 2).

Food Consumption

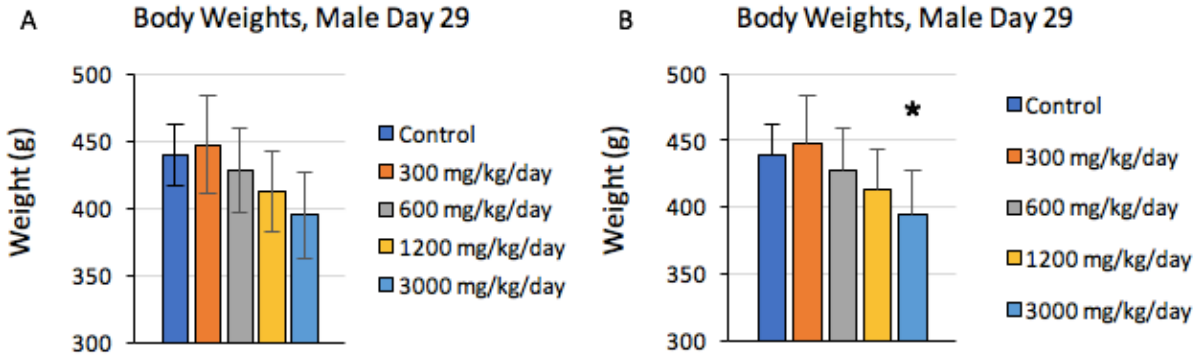
Males and females given 3000 mg polydatin/kg/day consumed less food during Week 1 of the dosing phase (Table 3). This decrease was statistically significant for males. Lower food consumption continued to be observed for males and females for Weeks 2 and 3 of the dosing phase; however, it was not statistically significant and the difference became smaller in magnitude with each week. During Week 4 of the dosing phase, food consumption for males and females given 3000 mg/kg/day was comparable with control rats. Lower food consumption was also observed during Weeks 1, 2, 3, and 4 (females only) of the dosing phase for animals given 600 or 1200 mg/kg/day (Table 3). However, this was not statistically significant. This decrease was considered polydatin-related given that a correlating decrease in body weight was observed for these animals. Due to the absence of relevant clinical observations and small magnitudes of the changes, decreases in food consumption were not considered adverse.

Figure 1[1]



Weight gain of rats based upon polydatin dose over the course of the experiment. Rats received their first dose of polydatin on Day 1. (A) Female rats, n=10 (B) Male rats, n=10 per group. Results are shown as mean ± stdev.[1]

Figure 2[1]



Body weights on Day 29 prior to study termination. A) Female rats, n=10 per group; B) Male rats, n=10 per group. * P<0.05 compared to control. Results are shown as mean ± stdev. [1]

Table 2[1]

Percent Decrease in Body Weight

Group	Dose Level (mg/kg/day)	Males				Females			
		Day 8	Day 15	Day 22	Day 29	Day 8	Day 15	Day 22	Day 29
2	300	-	-	-	-	-	-	-	-
3	600	-	1.6	1.5	2.5	-	2.6	2.9	5.0
4	1200	4.3	5.3	5.9	5.9	1.4	2.6	3.7	6.6
5	3000	6.8*	7.5*	9.1*	10.0*	2.4	4.4	6.6	8.1

- = No noteworthy differences.* = Statistically significant at P <= 0.05.
Body weight for animals given 300 mg/kg/day as comparable with controls.[1]

Table 3[1]

Percent Decrease in Food Consumption

Group	Dose (mg/kg/day)	Males				Females			
		Week 1	Week 2	Week 3	Week 4	Week 1	Week 2	Week 3	Week 4
1	0	-	-	-	-	-	-	-	-
2	300	-	-	-	-	-	-	-	-
3	600	2.3	3.6	2.2	1.9	1.4	2.0	3.0	4.5
4	1200	6.5	3.6	3.5	-	6.8	7.9	7.8	5.7
5	3000	11.1*	6.7	4.4	-	10.2	7.9	6.6	3.2

- = No noteworthy differences.* = Statistically significant at P <= 0.05.
Food Consumption (% lower compared with control)[1]

Toxicokinetic Analyses

Polydatin

Mean concentration time profiles for males and females showed mean concentrations of polydatin were generally similar after a single dose and multiple doses of polydatin (Table 4). After oral administration, polydatin was readily absorbed, with Tmax values ranging from 0.500 to 1.00 hours on Days 1 and 25 of the dosing phase. Polydatin was readily eliminated, and concentration values for polydatin were below the limit of quantitation by 12 hours postdose on Days 1 and 25 of the dosing phase.

Table 4[1]

Toxicokinetic Parameters for Polydatin and *Trans*-Resveratrol in Rat Plasma

Analyte	Polydatin Dose Level (mg/kg/day)	Day	Sex	C_{max} (ng/mL)	T_{max} (hr)	AUC_{0-t} (ng·hr/mL)	AUC_{0-24} (ng·hr/mL)	$AUC_{0-\infty}$ (ng·hr/mL)	M/P AUC_{0-24} Ratio
polydatin	300	1	M	2857	1.00	9699	10291	NC	
			F	3183	0.500	10640	10964	11003	
		25	M	2690	1.00	7629	7741	NA	
			F	4700	0.500	9472	9593	NA	
trans-resveratrol	300	1	M	467	2.00	3235	3619	NC	0.352
			F	333	2.00	2064	2139	NC	0.195
		25	M	302	2.00	2171	2773	NA	0.358
			F	292	0.500	1630	1827	NA	0.190

NA = Not applicable; NC = Not calculated.[1]

Cmax and AUC_{0-24} values for polydatin were higher for females than for males, but the differences were less than 2-fold. Values for Cmax and AUC_{0-24} were lower on Day 25 compared with Day 1 of the dosing phase, indicating no accumulation of polydatin after multiple dosing.

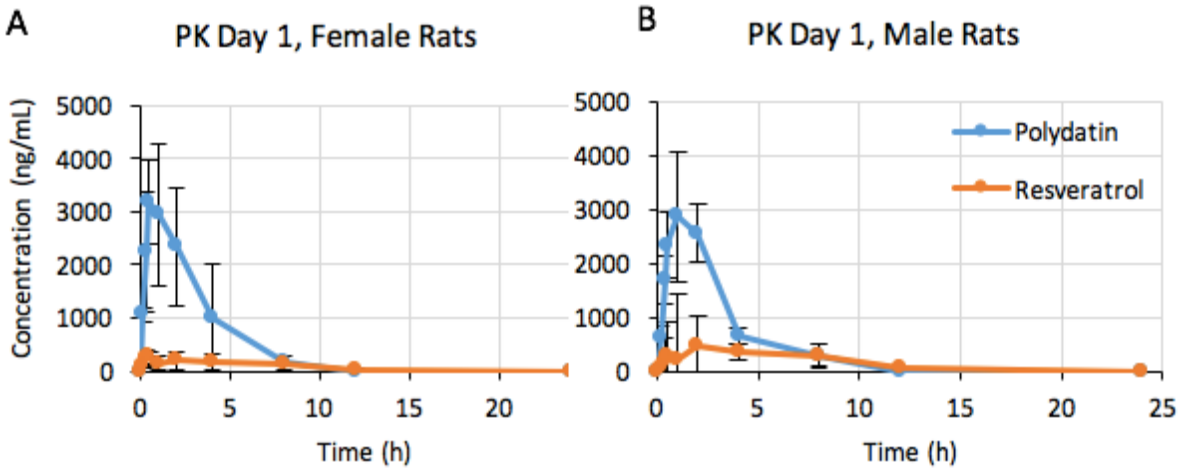
Trans-Resveratrol

Mean concentration time profiles for males and females showed mean concentrations of trans-resveratrol were generally similar after a single dose and multiple doses of polydatin (Table 4). After oral administration, trans-resveratrol readily appeared in the plasma, with Tmax values of 2.00 hours on Days 1 and 25 of the dosing phase. Plasma levels readily declined and were below the limit of quantitation by 24 hours post-dose on Days 1 and 25 of the dosing phase. Cmax and AUC_{0-24} values for trans-resveratrol were higher for males than for females, but the differences were less than 2-fold. Values for Cmax and AUC_{0-24} were lower on Day 25 compared with Day 1 of the dosing phase, indicating no accumulation of polydatin after multiple dosing. Mean AUC_{0-24} ratios of trans-resveratrol to polydatin (metabolite-to-parent ratio) were 0.352 and 0.358 for males and 0.195 and 0.190 for females on Days 1 and 25 of the dosing phase, respectively.

Although maximum serum levels of polydatin were slightly less in males than in females, both sexes absorbed polydatin with similar kinetics, reaching peak levels (approximately 3.0 mg/ml) within 1 hr of dosing (Figure 3). Serum trans-resveratrol levels were 10-fold less than polydatin and attained maximal levels in both male and females approximately 2 hr after dosing. Importantly, the levels of both polydatin and trans-resveratrol were below the limit of detection 24 hr after dosing. Similar results were also found on day 25 of the toxicokinetic study indicating that there was no accumulation of polydatin or resveratrol after repeat dosing.

Figure 3[1]

Pharmacokinetic Concentrations of Polydatin and Trans-Resveratrol



Serum concentrations of polydatin and trans-resveratrol after the oral gavage of 0.3 g/kg of polydatin in female (A) and male (B) rats. Nine animals per sex were used with blood samples collected from three animals/sex/time point. Graphs represent the results obtained on the first day of dosing. The levels of polydatin and trans-resveratrol were assumed to be 0 at time zero because the rats had not been previously exposed to polydatin. Results are shown as mean ± standard deviation. [1]

Clinical Pathology, Hematology and Coagulation

No polydatin-related effects were observed in hematology and coagulation data.

Minimally shortened prothrombin time in males given >1200 mg/kg/day was of small magnitude (≤0.5 sec) and, considering the normal variability in coagulation times, was not considered test article-related or toxicologically important. Shortened prothrombin time was slightly surprising as others have found polydatin protects again thrombosis possibly by decreasing platelet-neutrophil interactions. [62]

Clinical Chemistry

Several minor polydatin-related findings were observed in clinical chemistry test[63] results but they were of small magnitude and not considered adverse or toxicologically important. Findings at >600 mg/kg/day included the following.

- Minimally lower glucose in males given >600 mg/kg/day and females (not statistically significant) given 3000 mg/kg/day
- Minimally higher albumin in males given >1200 mg/kg/day
- Minimally lower globulin and higher albumin-to-globulin ratio in males given >600 mg/kg/day
- Minimally higher ALT activity in males and females given >600 mg/kg/day
- Minimally higher alkaline phosphatase (ALP) activity in females given >600 mg/kg/day
- Minimally lower potassium in females given 3000 mg/kg/day

Minimally higher alanine aminotransferase (ALT) activity in males was the only clinical chemistry finding at 300 mg/kg/day. Overall, elevations in ALT (1.2 to 2.1x control) and ALP (1.3 to 1.6x control) were of small magnitude and not associated with microscopic findings in the liver. No rise was detected in total bilirubin, also a strong indicator of drug induced liver injury (DILI). Other studies have shown polydatin to have a protective hepatic mechanism in mice dosed up to 300mg/kg[64] and 100 mg/kg.[65]

Figure 4[1]

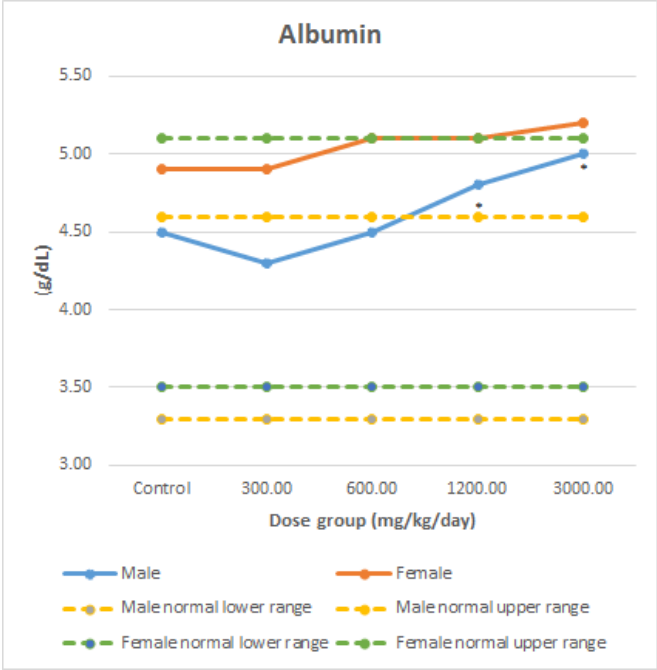
Glucose Serum Levels

Glucose serum levels of male and female rats on Day 29. Female rats, n=10 per group; Male rats, n=10 per group. *= Statistically significant at P <= 0.05.[1] Normal value range based on Charles River historical data of Crl:CD(SD) Rats.[63]

Lower glucose (see Figure 4) and potassium may have been associated with reduced feed consumption observed in these animals. However, as we discussed earlier, previous studies have identified polydatin as capable of lowering glucose levels in diabetic models of mice[40] and rats[13] by stimulating insulin secretion. High albumin (See Figure 5) commonly occurs with dehydration, however, dehydration was not evident clinically or in other clinical pathology data (serum urea nitrogen and creatinine). It should be noted that lower albumin levels are more often associated with liver disease,[66] nephrotic syndrome,[67] and malnutrition.[68]

Figure 5[1]

Albumin Serum Levels



Albumin serum levels of male and female rats on Day 29. Female rats, n=10 per group; Male rats, n=10 per group. *= Statistically significant at P <= 0.05.[1] Normal value range based on Charles River historical data of Crl: CD (SD) Rats.[63]

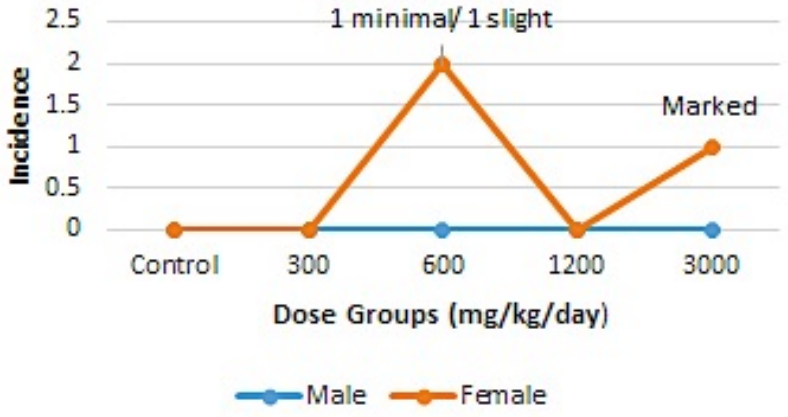
Urinalysis

None of the urinalysis findings were considered adverse or toxicologically important. Minimally lower urine pH in males and females given >1200 mg/kg/day and higher incidence or severity of urine occult blood and presence of red blood cells in the urine of a few males given 3000 mg/kg/day and presence of white blood cells in the urine of a few females given 3000 mg/kg/day were considered polydatin-related findings but not adverse. The change in pH appeared consistently present in most animals, but the remaining urinalysis findings were observed in individual animals and most were not associated with correlative microscopic findings.

One female given 3000 mg/kg/day had increased severity of white blood cell in the urine, which correlated with marked chronic active pelvic inflammation observed microscopically in the kidneys (see Figure 6). However, one male given 1200 mg/kg/day with microscopic evidence of marked acute renal hemorrhage had no occult blood or presence of red blood cells in the urine. Additionally, two females given 600 mg/kg/day with microscopic findings of chronic active inflammation in renal pelvis (see Figure 6) had no increase in evidence or severity of white blood cells in the urine, although another animal had presence of red blood cells in the urine.

Figure 6[1]

Chronic Active Pelvic Inflammation

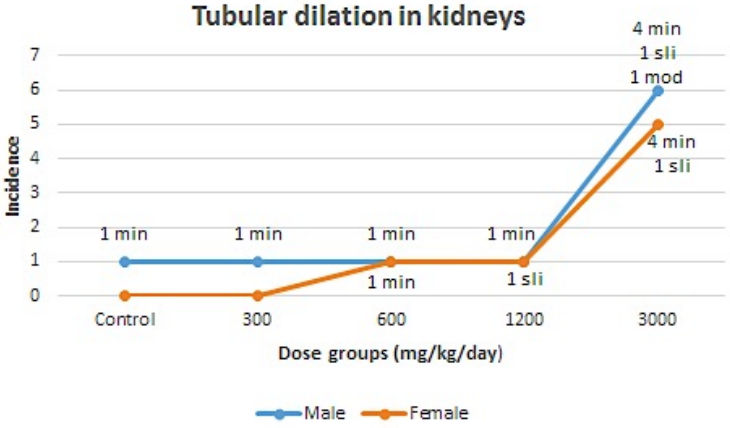


Chronic active pelvic inflammation in male and female rats. Female rats, n=10 per group; Male rats, n=10 per group.[1]

Test results of interest included increased incidence and severity of tubular dilatation (see Figure 7), increased incidence and severity of hyaline (protein) droplets in the tubule cells (males only) (see Figure 8), erosion/ulceration of the transitional epithelium (see Figure 9), and inflammation of the renal pelvis (see Figure 6).

Figure 7[1]

Tubular dilation in kidneys

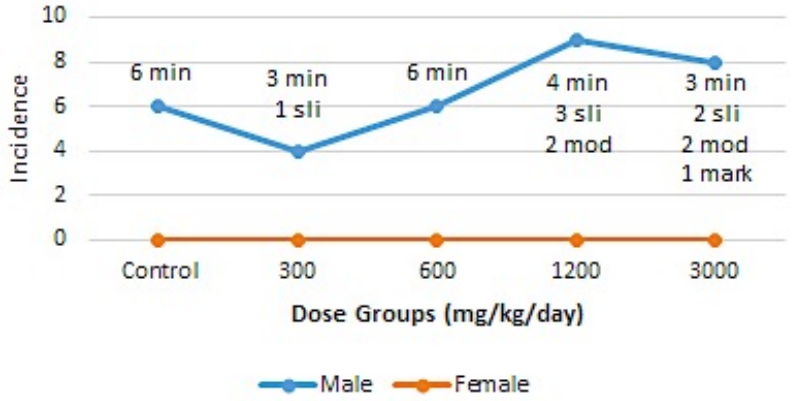


Tubular dilation in kidneys in male and female rats. Female rats, n=10 per group; Male rats, n=10 per group. *Abbreviations:* min-minimal; sli-slight; mod-moderate.[1]

Statistically significant or otherwise notable differences observed for other clinical pathology test results were considered incidental because they were usually of very small magnitude and/or lacked a relationship to dose.

Figure 8[1]

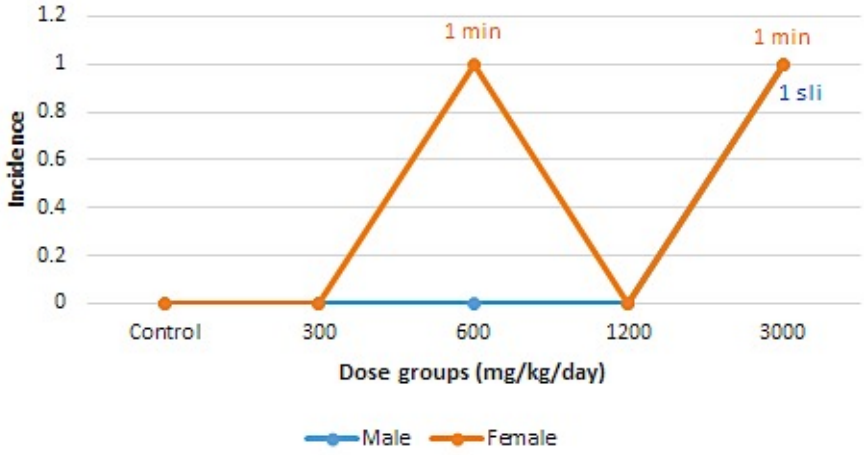
Hyaline Droplets in the Tubule Cells



Hyaline droplets in the tubule cells of male and female rats. Female rats, n=10 per group; Male rats, n=10 per group. *Abbreviations:* min-minimal; sli-slight; mod-moderate; mark-marked.[1]

Figure 9[1]

Ulcer/Erosion of the Transitional Epithelium



Ulceration of the transitional epithelium in male and female rats. Female rats, n=10 per group; Male rats, n=10 per group. *Abbreviations:* min-minimal; sli-slight.[1]

Anatomic Pathology

Polydatin related terminal body weight changes were noted in animals given 3000 mg/kg/day compared with controls. Mean terminal body weight was 10% lower in males and 8% lower in females; the change was only significant in males. Brain-to-body weight and kidney-to-body weight ratios were significantly higher in males given 3000 mg/kg/day, which were attributed to the lower terminal body weight. Absolute liver weight in males given 3000 mg/kg/day was significantly lower (12.9%) than controls; these findings were considered spurious since no microscopic correlate was present.

Absolute and relative mean spleen and thymus weights were also lower in males and females given 3000 mg/kg/day, though not significantly. There was no microscopic correlate for the spleen or thymus weight changes; they were attributed to biological variation. Individual spleen weights did not correlate with the lymphocyte depletion noted below.

Macroscopically, at 3000 mg/kg/day, large cecum was noted in 2/10 males and 3/10 females, and a large colon was noted in 1/10 males. At 1200 mg/kg/day, a large cecum was noted in 1/10 females. This finding was considered test article-related, although no microscopic correlate was present.

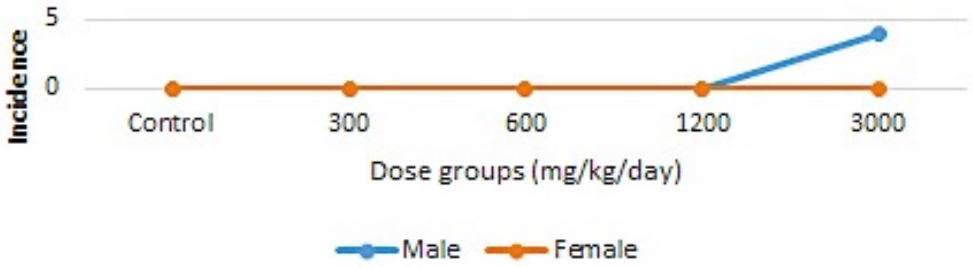
Polydatin-related microscopic kidney findings were limited to males given >1200 mg/kg/day and females given 600 or 3000 mg/kg/day. Kidney findings were sporadic, varied between animals, and did not often show a clear dose response relationship. The only microscopic finding that was considered adverse was the marked bilateral chronic active inflammation and transitional cell hyperplasia in one female given 3000 mg/kg/day (see Figure 6). Although chronic inflammation was only noted in the aforementioned animal, it was considered test article-related because of the presence of minimal to slight acute or chronic/active inflammation in several other treated animals given lower doses, the marked severity of the lesion, and the lack of evidence of any other inciting cause (i.e., a calculus causing chronic irritation). Similar inflammation in two females given 600 mg/kg/day was not considered adverse due to the lower severity and the unilateral nature of the inflammation. The hyaline protein droplets in the tubular cells of the males were also not considered adverse, even at a marked severity, because there was no clinical pathology evidence of renal protein loss.

An unusual finding of uncertain relationship to the test article was the presence of marked acute hemorrhage in the kidney of one male given 1200 mg/kg/day. The cause of the hemorrhage was not evident in examined sections. This animal also was noted to have a urinary bladder that was discolored and filled with red fluid at necropsy.

Lymphocyte depletion was observed in the mantle zone of the spleens in 4/10 males in the test article group given 3000 mg/kg/day (see Figure 10). This finding was not considered a primary test article related effect, and was attributed to stress. Lymphocyte depletion was not seen in any other examined lymphoid organs (thymus, mesenteric lymph node, mandibular lymph node, and gastrointestinal lymphoid tissue present on examined sections of intestine).

Figure 10[1]

Depletion of Lymphocytes in Mantle Zone of Spleen



Depletion of lymphocytes in mantle zone of spleen in male and female rats. Female rats, n=10 per group; Male rats, n=10 per group.[1]

Discussion[1]

In this study, administration of polydatin up to 3000 mg/kg/day did not result in any adverse effects on the general health of the animals. Although administration of polydatin caused reductions in body weight, body weight gain, and food consumption, decreases in body weight and food consumption were of small magnitude. Furthermore, decreases in food consumption

were primarily noted during the first 3 weeks of dosing, suggesting animals were starting to adapt by Week 4 of the dosing phase.

Reduction in lymphocytes appears to be systemic and likely related to stress. One cortisol treatment has been shown to reduce lymphocytes by as much as 70% in humans.[69] Other occurrences in the study which could be interpreted as stress-related include changes in the spleen cellularity and lymph node cellularity in males, and increases in circulating neutrophils compared to control (although not significant).[70]

Other test related findings included minimally higher ALT in females dosed at ≥600 mg/kg/day but there was no increase in liver weight, or rate of hepatocellular changes denoting necrosis or degeneration of the bile duct greater than seen in the control group. Similar studies in humans have been reported in which weight loss in women was associated with an increase in ALT and AST but no hepatic histological changes were noted.[71]

Urinary related findings included dilated tubules, presence of hyaline droplets, dilation of the renal pelvis, inflammation of the pelvis, and hyperplasia and or ulceration of the transitional epithelium. Dilated tubules may be regarded as normal if present in small amounts.[72] Hyaline droplets are also of little concern if restricted to younger male animals as seen in the study and appear in equal rates in the treated and control groups.[73] The only adverse test article-related finding noted in one female given 3000 mg/kg/day was chronic active pelvic inflammation and transitional cell hyperplasia. Based on these results, the no observed adverse effect level (NOAEL) is 1200 mg/kg/day for females and 3000 mg/kg/day for males.

All animals in the toxicokinetic phase of the study (300 mg/kg/day) were exposed to polydatin and its metabolite, trans-resveratrol as verified by the presence of these two materials in the plasma samples. No marked sex differences were observed in polydatin and trans-resveratrol Cmax and AUC_{0-24} values. No accumulation of polydatin or trans-resveratrol was observed after multiple dosing of polydatin.

Conclusion

All animals survived to the end of the study. No polydatin-related clinical observations or ophthalmic findings were noted. No marked sex differences were observed in polydatin Cmax and AUC_{0-24} values. No accumulation of polydatin was observed after multiple dosing. The only adverse test article-related finding noted in one female given 3000 mg/kg/day was chronic active pelvic inflammation and transitional cell hyperplasia. Based on these results, the no observed adverse effect level (NOAEL) is 1200 mg/kg/day for females and 3000 mg/kg/day for males.

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Appendix E

A Phase 1 Clinical Pharmacokinetic Study of BSN272 in Healthy Volunteers

Protocol #XXXXXX-001

Study Product: BSN272
Clinical Sites: University of Kentucky
Clinical laboratory:
IND Number: XXXXXX

Initial DRAFT Version: 1

Study Summary

Title	An exploratory phase 1 clinical pharmacokinetic study of BSN272 in healthy volunteers.
Short Title	Pharmacokinetic, single dose study of BSN272 in healthy volunteers.
Protocol Number	XXXXXX-001
Phase	Phase 1
Methodology	2-arm single dose of trans-polydatin (100 µg) and D-lyxo-hexulose (0.03 g/kg) pharmacokinetic study over 24 hours.
Study Duration	24-36 hours
Study Center(s)	University of Kentucky
Objectives	To test the hypothesis that the t_{max} and c_{max} of a single dose of trans-polydatin (100 µg) does not change when administered with a single dose of D-lyxo-hexulose (0.03 g/kg).
Number of Subjects	Screen up to 32 to enroll a total of 16 healthy subjects (18-30 yrs. old), 4 alternates and 12 needed to complete the trial.
Diagnosis and Main Inclusion Criteria	Healthy Volunteers
Study Product, Dose, Route, Regimen	Single oral dose of trans-polydatin (100 µg) and D-lyxo-hexulose (0.03 g/kg) in a 2-arm study.
Duration of administration	Single dose
Reference therapy	None
Statistical Methodology	A t-test (p=0.05) will be used to calculate the significance of the difference between the C_{max} and T_{max} of t-PD with and without DLH.

Table of Contents

Abbreviations:

AE	adverse event
apoB	apolipoprotein B
ALT	Alanine transaminase
BSN272	D-lyxo-hexulose + trans-polydatin combination
BUN	blood urea nitrogen
CO2	carbon dioxide
CRF	case report form
CRO	contract research organization
DRF	Discrepancy Resolution Form
DLH	D-lyxo-hexulose (also known as D-tagatose)
EDI	estimated daily intake
ERB	Ethics Review Board
EU	European Union
FDA	Food and Drug Administration
FH	familial hyperlipidemia
FCHL	familial combined hyperlipidemia
GRAS	generally recognized as safe
HbA1c	glycated hemoglobin
HepC	hepatitis C
HIV	human immunodeficiency virus
ICH	International Conference on Harmonisation
IDL	intermediate-density lipoprotein
IRB	Institution Review Board
LDL	low density lipoprotein
MCH	mean corpuscular hemoglobin
MCHC	mean corpuscular hemoglobin concentration
MCV	mean corpuscular volume
MR	magnetic resonance
OHRP	Office for Human Research Protection
PEA	phenylethylamine
PK	pharmacokinetic
SAE	severe adverse event
SGOT	serum glutamic-oxaloacetic transaminase
SPGT	serum glutamic-pyruvic transaminase
TG	triglyceride
t-PD	trans-polydatin
VLDL	very-low-density-lipoprotein
WBC	white blood cell
WHO	World Health Organization

Abstract

The primary objective of this phase 1 exploratory, microdose study is to determine whether the t_{max} and C_{max} of trans-polydatin (t-PD) change significantly when coadministered with D-lyxo-hexulose (DLH) in healthy male and female volunteers over a 24-hour period. BSN272 is a potential drug therapy for the treatment of persons with Prader Willi Syndrome (PWS). Healthy volunteers will be administered doses of trans-polydatin or BSN272. Blood samples will be taken pre-dose (t = 0) and at approximately 0.167 (10 min), 0.333 (20 min), 0.5, 1, 2, 4, 6, 8, 12 and 24-36 hours post drug administration. Urine samples will also be collected during the 12 hour onsite clinical period, with one subsequent sample collected at visit 3, which should approximate the 24 hour post-treatment period. Serum and urine samples will be assayed for trans-polydatin and the metabolite trans-resveratrol at each time point to determine the pharmacokinetic effects of the combination BSN272 drug.

Introduction

Background:

Prader-Willi Syndrome

Persons with Prader-Willi Syndrome (PWS) are at risk of premature death (Einfeld, et al., 2006; Stevenson, et al., 2007). In one study using the demographic data on 178 deceased individuals with PWS, the median and average age at death was 27 years and >25% of individuals were <19 years (Stevenson, et al., 2007). In a second study of 36 adults with PWS in Australia, there were 10 deaths at a mean age of 33 years (Smith, 2003). PWS is the most commonly known genetic cause of life-threatening obesity. Estimates of the prevalence of PWS range between 1 in 12,000 to 1 in 52,000, with the most likely figure being 1:15,000 (Burd, et al., 1990; Whittington et al., 2001: www.pwsausa.org). One of the characteristics of PWS is an insatiable appetite resulting in obesity. Obesity is associated with an increased risk for diabetes and cardiovascular disease and markers for these obesity related diseases, such as insulin resistance and dyslipidemia, are commonly exhibited by patients with PWS. Because of the complex nature of PWS, and the multiple symptoms exhibited by patients with PWS**,** there is currently no single drug available that can successfully treat all of the symptoms. For example, growth hormone can increase stature and muscle tone, but does not affect hyperphagia. D-lyxo-hexulose (DLH, also known as D-tagatose) has been shown in both animal and human studies to have a beneficial effect on many of the symptoms exhibited by PWS patients including satiety, weight control, blood lipids, atherosclerosis, and type 2 diabetes mellitus. Studies with trans-polydatin indicate positive results for a number of health issues that commonly impact patients with PWS, such as preventing diet induced increases in cholesterol and triglycerides (Arichi et al 1982; Du et al 2009; Xing et al 2009). Studies have shown that the combination of DLH and t-PD is synergistic, and have even shown that undesirable side effects of one can be eliminated by the

other (Lodder et al 2015). **This makes BSN272, the combination of D-lyxo-hexulose (DLH) and trans-polydatin (t-PD), an excellent drug candidate for the treatment of patients with PWS.**

PWS is a complex multisystem genetic disease characterized by hypothalamic-pituitary dysfunction with varying degrees of penetrance in humans. Hypothalamic dysfunction has been implicated in many manifestations of this syndrome including hyperphagia, temperature instability, high pain threshold, sleep disordered breathing, and multiple endocrine abnormalities. These endocrine abnormalities include growth hormone deficiency with resulting short stature, central adrenal insufficiency, hypogonadism, hypothyroidism, central obesity and complications of obesity such as type 2 diabetes mellitus (Emerick and Vogt, 2013). Multiple studies have indicated high prevalence of central adrenal insufficiency (CAI) (Corrias et al., 2012) and CAI as the apparent cause of sudden deaths in PWS patients (de Lind van Wijngaarden, 2008). The evidence presented above has led some to hypothesize hypothalamic-pituitary-adrenal (HPA) axis dysfunction as the cause of PWS (Farholt et al., 2011).

The major medical concern for patients with PWS is morbid obesity. Compulsive eating and obsession with food usually begins between the ages of 1 and 6. The urge to eat is physiological and overwhelming; it is difficult to control and requires constant vigilance. Weight control depends on food restriction and daily exercise. Currently there is no medication or surgical intervention that can eliminate the need for strict dieting and supervision around food. Studies have repeatedly shown that PWS patients are at risk of premature death. Mortality in children is most commonly associated with respiratory infection and high temperature resulting in sudden death. Common in adults with PWS are obesity-related problems such as respiratory failure and pulmonary hypertension, obstructive sleep apnea, hypertension, and type 2 diabetes mellitus (T2DM) (Einfeld, et al., 2006; Lionti, 2012; Whittington, et.al., 2001; Vogels, et. al., 2004). The cause of death in adults is usually related to failures of the circulatory or respiratory systems.

A high incidence of alterations in glucose metabolism including impaired fasting glucose, impaired glucose tolerance, and T2DM, has been observed in patients with PWS, particularly after adolescence (Butler et al., 2002). Mean age at onset of diabetes is about 20 years of age. A prevalence rate of 20 to 25% for T2DM is found in patients with long standing PWS (Butler *et al*., 2002; Donaldson et al., 1994).

Obesity and diabetes are well-established risk factors for premature cardiovascular disease. A limited number of studies have looked at cardiovascular fitness and disease markers in PWS patients. Cardiovascular risk factors are evident in a large percentage of children with PWS. de Lind van Wijngaarden et al. (2010) looked at the cardiovascular and metabolic risk profile in 85 children with Prader-Willi syndrome (PWS). They found that infants and prepubertal children had at least one cardiovascular risk factor. In 63% of the infants and 73% of prepubescent children, at

least one of the following factors was present: elevated systolic or diastolic blood pressure; elevated serum total cholesterol, low density lipoprotein cholesterol, triglycerides, or lipoprotein(a) levels; or reduced high density lipoprotein cholesterol levels in addition to a high fat%, indicating an unfavorable cardiovascular profile. Patel, et al. (2007) assessed cardiac and vascular structure and function in nine PWS patients and found significantly elevated high-sensitivity C-reactive protein (hs-CRP), (hs-CRP has been shown to be significantly elevated in patients dying suddenly with severe coronary artery disease (Burke, et al., 2002)), and evidence of microcirculatory dysfunction as evidenced by decreased peak hyperaemic flow response. In a small study involving 36 adults with PWS who were followed for 10 years there were 10 deaths (Smith, 2003). The average age at death was 33.2 years with 60% of the deaths due to cardiovascular reasons including "strokes, coronary occlusion, or heart failure". Lionti, et al. (2012) also concluded that cardiac or respiratory conditions were common causes of death of those with PWS after the age of 15 years.

D-lyxo-hexulose

Many of the problems faced by those afflicted with PWS stems from their struggle with obesity. Obesity is associated with an increased risk for diabetes and cardiovascular disease, and markers for these obesity related diseases are exhibited by patients with PWS. D-lyxo-hexulose has been shown in both animal and human studies to have a beneficial effect on many of the symptoms exhibited by PWS patients (although there have not been any studies of D-lyxo-hexulose in PWS to date) including lack of satiety, weight control, dyslipidemia, atherosclerosis, and type 2 diabetes mellitus (Tables 1-4).

Table 1. D-lyxo-hexulose Effect Upon Satiety

Type of Study	Species	Dose Level	Results	Reference
Human pilot study	Humans (4♂4 ♀ with T2DM)	15 g t.i.d.-1 year duration	Satiety was reported as a side effect in 3 subjects early in the experiment.	Donner et al 2010
Human double blind cross-over	Normal weight humans (3♂5♀)	30 g QD-2 wk duration	Fullness evaluated 2.5 h after dinner showed an increase from 39%-52% compared to placebo (P=0.02). Response similar on Days 1&15.	Buemann et al. 1998
Human double blind cross-over	Healthy Humans (25♂25♀)	20 g QD-3 days over a 17 day duration	D-lyxo-hexulose was associated with significantly more thirst ($P < 0.01$) and appetite loss. Significantly more subjects reported grade 2 or 3 thirst and appetite loss ($P < 0.05$).	Lee and Storey 1999
Human double blind cross-over	Healthy humans (19♂)	29 g QD	Energy intake was 15% lower after D-lyxo-hexulose consumption compared to sucrose after supper ($P < 0.05$).	Buemann et al 2000b
Toxicology study	Crl: CDBR rats (20 rats/group/sex	Doses of 5, 10, 15, and 20%	A statistically significant reduction in mean weekly food consumption relative to the isocaloric control group for a large proportion of weeks.	Kruger et al 1999c

	)	D-lyxo-hexulose for 90 days		
Mouse study	50 male ApoE -/- (C57BL/6 background)	8 weeks	Energy consumption for the D-lyxo-hexulose group was 11.65 ± 1.33 Kcal/ day vs 15.77 ± 1.13 Kcal/day for mice on the western diet. ($P < 0.001$).	Unpublished report

Table 2. D-lyxo-hexulose Effect Upon Weight Control

Type of Study	Species	Dose Level	Results	Reference
Human Phase 2 clinical trial	161 randomized humans	2.5 g, 5.0 g, and 7.5 g t.i.d. for 6 mo	Mean body weights at baseline, 3-months, and 6-months for the 5.0 g D-lyxo-hexulose dose group were 165.4 ± 33.0 lbs, 161.5 ± 29.5 lbs, and 161.7 ± 27.5 lbs, respectively; and the mean body weights for the 7.5 g D-lyxo-hexulose dose group were 167.4 ± 33.0 lbs, 165.1 ± 30.5 lbs, and 160.6 ± 29.3 lbs, respectively.	Ensor et al. 2014
Human pilot study	Humans (4♂4 ♀ with T2DM)	15 g t.i.d.- 1 yr duration	Mean body weight decreased from 108.4 kg to 103.3 kg (P=.001).	Donner et al 2010
Rat toxicology study	80 Sprague Dawley male Crl:CD BR rats	20% D-lyxo-hexulose for 28 days	Body weights were significantly reduced in the 20% D-lyxo-hexulose groups compared to control.	Bar et al 1999
Rat toxicology study	Sprague-Dawley rats	15% D-lyxo-hexulose for 60 days	> 7% less weight gain for D-lyxo-hexulose-fed rats than controls. Sucrose-fed rats gained an average of 86 g as opposed to 28 g gained by the rats fed D-lyxo-hexulose.	Levin et al 1995
Rat toxicology study	Crl:CDBR rats (20 rats/group/sex)	Doses of 5, 10, 15, and 20% D-lyxo-hexulose for 90 days	Mean body weight values were statistically significantly decreased, compared to controls, in the 20% dose-group males on Days 21–91, the 15% dose-group males on Days 42–70 and Day 84, and the 20% dose group females on Days 63, 84, and 91.	Kruger et al 1999c
Mouse study	LDLr-/- mice (6 mice sex/group) (backcrossed 10 times on a C57BL/6J)	16 week duration	Male body weights were 27 g for D-lyxo-hexulose groups vs 30 g for the control and 40 ± 2 g for the sucrose group. Female weights were 24 ± 1, 24 ± 1, and 31 ± 1g, for the D-lyxo-hexulose, control, and sucrose groups respectively.P < 0.01 compared to TAG.	Police et al 2009
Mouse study	50 male ApoE -/- (C57BL/6 background)	8 week duration	Body weights were 29.5 ± 1.4 g, 31.24 ± 2.7 g, 37. ± 4.6 g for the D-lyxo-hexulose, standard chow and western chow groups, respectively.	Unpublished study

Table 3. D-lyxo-hexulose Effect Upon Lipids

Type of	Species	Dose Level	Results	Reference

Study				
Human Phase 3 clinical trial	356 humans in the ITT population	15 g t.i.d. for 1 year	LDL differences in LS mean for the D-lyxo-hexulose ITT population at month 6 include a reduction of 4.8 vs. 0.96, Δ = 4.1 (p = 0.0211) at month 8 a reduction of 6.6 vs. 3.6, Δ = 3.1 (p =0.0100) and at month 10 a reduction of 7.1 vs. 4.2, Δ = 2.9 (p = 0.0057). Total cholesterol ITT reduction vs. baseline was -14.8 mg/dl at month 12 (p=0.005)	Ensor et al 2015
Human Pilot study	Humans (4♂4♀ with Type II DM)	15 g t.i.d.- 1 yr duration	HDL rose from 30.5 ± 15.8 to 41.7 ± 12.1 mg/dL (in 6 subjects not on lipid modifying medication) (P<.001).	Donner et al. 2010
Human double blind cross-over	Normal weight humans (3♂5♀)	30 g daily- 2 wk duration	Triglycerides measurements of D-lyxo-hexulose vs Sucrose d 1: 1.20 ± 0.13 vs. 1.38 ± 0.19; d 7: 1.37 ± 0.13 vs. 1.41 ± 0.17; d 15: 1.46 ± 0.22 vs. 1.70 ± 0.39 mmol/L (P = 0.04).	Buemann et al. 1998
Mouse Study	LDLr-/- mice (6 mice sex/group)(backcrossed 10 times on a C57BL/6J)	16 week duration	Male triglycerides were 162 ± 29, 110 ± 20, 822 ± 148 mg/dl for the D-lyxo-hexulose, control, and sucrose groups, respectively. Female triglycerides were 54±8, 79 ± 16, and 326 ± 37 mg/dl for the D-lyxo-hexulose, control, and sucrose groups, respectively (P < 0.01).	Police et al. 2010
Mouse study	50 male ApoE -/- (C57BL/6 background)	8 week duration	By day 71, triglyceride results were less than 1/2 of either the Standard or Western diet groups (Standard diet, 263.7 ± 33.1 mg/dL; Western diet, 256.0 ± 31.9 mg/dL; D-lyxo-hexulose, 102.9 ± 5.65 mg/dL)	Unpublished report

Table 4. D-lyxo-hexulose Effects Upon Type 2 Diabetes Mellitus

Type of Study	Species	Dose Level	Results	Reference
Human pilot trial	Humans (8 normal and 8 with T2DM)	75 g D-lyxo-hexulose	Lower postprandial increases in blood glucose and insulin. Pre-OGTT treatment with 75 g D-lyxo-hexulose attenuated the rise in glucose levels in patients with DM (p < 0.02 at 60 and 180 min, and p < 0.01 at 120 min). The glucose area under the curve (AUC) was reduced significantly also by pre-treatment with D-lyxo-hexulose in a dose-dependent manner in patients with DM (p < 0.05 for 10 g D-tag, p < 0.001 for 20 g D-tag, and p = 0.0001 for 30 g D-tag).	Donner et al. 1999
	Humans (10 T2DM patients)	0,10 g, 15 g, 20 g, and 30 g of D-lyxo-hexulose	A 3-hour glucose AUC of 20,152 ± 6125 mg/dl/min following a 75-g OGTT, compared with pretreatment with D-lyxo-hexulose reduced the glucose AUC to 17,510 ± 4811 mg/min/dl with 10 g D-lyxo-hexulose (p < 0.05), to 15,779 ± 5703 mg/min/dl with 20 g D-tag (p < 0.001) and to 15,018 ± 5461 mg/min/dl with 30 g D-lyxo-hexulose	

			(p = 0.0001).	
Human pilot	Human (8 subjects with T2DM patients completed the study)	15 g D-tag t.i.d. for 1 year	Decrease in GlyHb was observed in the 8 subjects who completed the study from 11.2% ± 2.0% to 9.5% ± 2.0%. Three patients were given rescue medications.	Donner et al 2010
Phase 3 clinical trial	356 humans in the ITT population	15 g t.i.d. for 1 year	For the ITT population better reductions in fasting blood glucose levels from baseline were observed in the D-lyxo-hexulose group compared to the placebo group at all post baseline time points(month 6 (reduction of 2.0 vs. an increase of 4.1, Δ = 6.0 and p =0.0440), month 8 (reduction of 0.44 vs. an increase of 2.5, Δ 2.9 and P =0.0340), and month 10 (reduction of 0.25 vs. an increase of 6.6, Δ = 6.9 and P = 0.0079).	Ensor et al 2015

Trans-polydatin

Studies have presented evidence that trans-polydatin has many positive health effects on conditions that are commonly encountered by patients with PWS. Many of the problems faced by those afflicted with PWS stems from their struggle with obesity. Obesity is associated with an increased risk for diabetes and cardiovascular disease, and markers for these obesity related diseases are exhibited by patients with PWS. Multiple trans-polydatin studies indicate positive results in the following areas likely to impact patients with PWS: anti-inflammatory actions (Ji et al 2012; Cobellis et al 2011), cardioprotective activities (Du et al 2009; Xing et al 2009; Zhang et al 2014; Deng et al 2012; Liu et al 2012), protection against ischemia/reperfusion injury (Cheng et al 2006; Zhang et al 2008), benefits for congestive heart failure (Gao et al 2010), and prevention of fatty liver disease and insulin resistance (Zhang et al 2015). Additionally, trans-polydatin can regulate glucose and lipid metabolism (Hao et al 2014).

Table 5. Trans-Polydatin in Preclinical Hyperlipidemia

Reference and Purpose	Model system and Route of Administration	Dosing/Dose range [Source/Purity]	Results
Arichi H et al., 1982 To study effects of trans-polydatin	Male Wistar-King rats, 150 g; 7 rats/group Male ddY strain mice, ~20 g; 8 mice/group Mice-Intraperitoneal	Rats were given a standard diet and an oral dose of 0 or 10 mL/kg corn oil containing 10% cholesterol and 1% cholic acid for 7 days. In subgroups of 7 animals receiving oil, one group received 50 mg trans-resveratrol/kg body weight (bw) and 2 groups were given either 50 or 100 mg/kg bw piceid 30 min prior to oil intubation. Study duration 7 days.	7 day rat study: Both RES and piceid were unable to prevent diet induced increases in levels of total cholesterol, free fatty acids or phospholipids, or prevent reductions in HDL-cholesterol levels with significance compared to positive control. However, piceid significantly prevented accumulation of LDL-ch (both doses) and rises in TG (100 mg/kg only) to the level of the positive control where RES did not. This resulted in a significantly lower atherogenic index for the 100 mg/kg

(piceid) and resveratrol (RES) on dietetically-induced hyperlipidemia	or oral Rat-Oral intubation (gavage)	Mice were fed a standard chow. RES groups received 25 mg/kg or 50 mg/kg. Piceid groups received 50 or 100 mg/kg. 14C-palmitate was injected 2 hours after the last injection of stilbenes. Study duration 3 days. RES or piceid were extracted and purified from Polygonum cuspidatum. Purity NS*.	piceid dosing group only, although not to the level of negative control. 3 day mouse study: Evaluation of lipogenesis in mice by measurements of incorporation of 14C-palmitate in liver and adipose tissue showed that 25 or 50 mg/kg RES given by i.p. injection or by oral gavage significantly ($p<0.001$ to 0.05) reduced lipogenesis in liver but only the highest dose of RES significantly ($p<0.05$) reduced lipogenesis in adipose tissue.
Du J et al., 2009 To evaluate the effects of polydatin (Piceid) in dietetically-induced hyperlipidemia	Male Syrian golden hamsters; 6 groups of 10 animals Dietary	One group of 10 hamsters was fed a control (normal fat) diet and 4 groups were fed high, fat-high cholesterol (2%) diet (HFC) and either 0, 25, 50 or 100 mg/kg bw polydatin; one additional group was given 10 mg/kg bw Fenofibrate as a positive (anti-lipidemic) control. Study duration: 15 days. [Polydatin isolated from Polygonum cuspidatum roots and purified by high speed, countercurrent chromatography; purity 91.3%]	HFC diet produced significant ($p<0.01$) increases in total cholesterol (TC), triglycerides (TG), HDL, and LDL cholesterol levels. In comparisons to the HFC animals, polydatin significantly prevented, diet-induced elevations in TC, TG and LDL levels at dosage-related levels. Polydatin did not significantly affect levels of HDL cholesterol in the range of doses studied. Polydatin significantly prevented diet-induced increases in LDL/HDL and TC/HDL ratios and in hepatic TG in comparisons to animals given HFC diet. The authors speculated that decreased lipid and cholesterol effects from ingestion of polydatin involve modulating roles in lipid metabolism and liver protection.
Xing W.W. et al., 2009 To determine effects of Polydatin on dietetically-induced hyperlipidemia	Male Japanese Giant Ear rabbits; 8 animals/group Dietary	Groups of rabbits were fed standard or high fat diets (HFD; 15% egg yolk, 1% cholesterol and 5% lard) for 3 weeks. Animals in the HFD group were then given oral, gavage doses of Polydatin of 0, 25, 50 or 100 mg/kg bw/day. Study duration = 3 weeks. Polydatin isolated and purified from Polygonum cuspidatum root; purity 91.3%.	HFD produced significant ($p<0.001$) increases in total cholesterol (TC), HDL, LDL and TC/HDL ratios in all groups compared to rabbits receiving normal diet. In comparison to HFD controls, Polydatin produced dosage-related, statistically significant ($p<0.05$ to $p<0.01$) decreases in TC, LDL and TC/HDL ratios but did not affect HDL levels in comparisons to the HFD control animals. The decrease in TC/HDL ratios in HFD animals given Polydatin suggested a cardioprotective role for Polydatin in modulating lipidemias associated with high fat diet. Other endpoints: There were no significant effects on body weight during the study in any of the treated groups

			compared to the control animals fed standard diets. However, the 50 and 100 mg/kg dosings were able to lower the liver weight coefficients compared to control.

*NS = Not specified

BSN272

Biospherics.net LLC has conducted a number of studies with BSN272 (combination of t-PD and DLH) in multiple animal models designed to evaluate the beneficial effects of preventing diet induced increases in cholesterol and triglyceride models. The results are summarized in Table 6 below.

Table 6. Oral Administration of BSN272 and Hyperlipidemia

Study Title and Purpose	Model System and Route of Administration, dose, duration	Endpoints	Results
BSN272 in apoE-deficient mice To evaluate the effect of a combination of trans-polydatin and D-lyxo-hexulose on development of genetically and dietetically induced hyperlipidemia and atherosclerosis	ApoE-deficient mice fed a diet high in carbohydrates, fats, and cholesterol Route of Administration: Dietary Dose: Control mice (n=14) were fed a diet containing 34% Sucrose, 21% milk fat, and 0.15% cholesterol BSN272-treated mice were fed 34% D-lyxo-hexulose, 21% milk fat, and 0.15% cholesterol Duration: 8 weeks	1. Serum Lipids 2. Body weight 3. Organ and fat deposit weight 4. Atherosclerotic lesion area 5. Food Intake	1. BSN272 significantly reduced serum cholesterol, VLDL cholesterol, and HDL cholesterol and although serum triglycerides and LDL cholesterol were reduced, the reductions were not statistically significant 2. BSN272 treated mice did not gain weight 3. The weight of epididymal, retroperitoneal, and subcutaneous fatty deposits was reduced in BSN272 treated mice compared to the control mice and there was no difference in the weights of the spleens, heart, and livers of the control and BSN272 treated mice. 4. BSN272 significantly reduced the size of the atherosclerotic lesions at the Sinus of Valsalva, Aortic Arch, and the Thorax. 5. Control and BSN272 treated mice consumed similar amounts of calories over the course of the 6 week treatment.
LDLr-deficient mice To evaluate the effect of a combination of trans-polydatin and D-lyxo-hexulose on development of genetically and dietetically	LDLr-deficient mice fed normal chow and gavaged with water, glucose and fructose, or uncorrelated doses of glucose, fructose, and D-lyxo-hexulose, or glucose, fructose, and BSN272 Route of Administration: Gavage	1. Serum Lipids during and after treatment (Blood was harvested from unfasted mice during treatment and from fasted mice at sacrifice (day 78)). 2. Atherosclerotic lesion area	1. Gavage of glucose and fructose significantly increased serum cholesterol on day 78, and free fatty acids on days 22 and 78. 2. Compared to those receiving glucose and fructose, gavage of uncorrelated mixtures of D-lyxo-hexulose, glucose, fructose significantly reduced serum cholesterol on day 36, triglycerides on days 36, 78, and free fatty acids on days 36, and 78 3. Compared to those receiving glucose and

induced hyperlipidemia and atherosclerosis	Dose: Control mice (n=14) were gavaged with water Glucose/Fructose-treated mice were gavaged with 25.6 g/kg Glucose and 25.6 g/kg Fructose/day Glucose/Fructose/D-lyxo-hexulose-treated mice were gavaged with uncorrelated doses of glucose, fructose, and D-lyxo-hexulose/day Glucose/Fructose/BSN272-treated mice were gavaged with uncorrelated doses of glucose, fructose, and BSN272/day Duration: 8 weeks	3. Body weight 4. Food Intake	fructose, gavage of uncorrelated mixtures of D-lyxo-hexulose, glucose, fructose SPX-106T significantly reduced cholesterol on days 36, 50, and 78, triglycerides on days 22, 36, and 78, and free fatty acids on days 14, 36, and 78 4. Compared to those receiving water, D-lyxo-hexulose significantly reduced triglycerides on day 78, and free fatty acids on days 14, 50 and 78. 5. Compared to those receiving water, BSN272 significantly reduced cholesterol on days 36 and 50, and triglycerides on day 78, and free fatty acids on days 14 and 78. 6. Compared to the group receiving, glucose and fructose, BSN272 significantly reduced VLDL, and LDL cholesterol at day 78 7. Compared to the group receiving glucose and fructose, BSN272 significantly reduced atherosclerotic lesion area at the aortic arch. 8. Neither D-lyxo-hexulose or BSN272 affected feed consumption or body weight gain.
Syrian Golden Hamsters To evaluate the effect of a combination of trans-polydatin and D-lyxo-hexulose on development of dietetically induced hyperlipidemia and atherosclerosis	Syrian Golden Hamsters fed a Western diet high in fats and carbohydrates Group 1 – positive control Group 2 – 6 g/kg BID tagatose Group 3 – 0.1 g/kg BID polydatin Group 4 – 6 g/kg tagatose + 0.025 g/kg polydatin BID Group 5 – 6 g/kg tagatose + 0.05 g/kg polydatin BID Group 6 – 6 g/kg tagatose + 0.1 g/kg polydatin BID	1. Serum triglycerides 2. Serum cholesterol	In all D-tagatose groups the drug was progressively run in to acclimate the animals. D-tagatose dose was reduced on day 10 of the study to 3 g/kg due to gastrointestinal disturbances. Although gastrointestinal intolerability to 12 g/kg/day of D-tagatose developed during the first days of dosing, all hamsters survived until the end of the study once the dose was reduced to 6 g/kg/day. Furthermore, combinations of D-tagatose and medium to high doses of polydatin reduced serum cholesterol, triglyceride, and free fatty acid levels more effectively than either component alone. Importantly, due to the lack of adverse events from day 11 to day 23, future studies should not exceed 6 g/kg/day without a proper run-in/adaptation period.

Current Treatment of PWS

The only FDA/EMA approved medication for PWS is somatropin, which is indicated for the pediatric population under the brand names Genotropin by Pfizer and Omnitrope by Sandoz. Somatropin is recombinant human growth hormone (rhGH). It is prescribed to combat below normal values of endogenous GH experienced by most patients with PWS. This has led to improvements in the health of children with regards to growth rate and height, physical strength, and body composition (Sanchez-Ortiga *et al*, 2012). However, growth hormone supplementation should not be used for the promotion of growth post fusion of the epiphysis according to the package insert for both Genotropin (Genotropin 2014) and Omnitrope (Omnitrope 2006). There is little evidence to suggest that the use of growth hormone in PWS has any major benefits in the adult population and it is our belief that the risks outweigh any possible benefit.

During a meta-analysis, eight studies were used to evaluate the use of growth hormone in adults with PWS (comprising a total of only 134 patients, 114 actually finished their respective trials) only 2 of which were randomized placebo controlled (Sanchez-Ortiga *et al* 2012). Analysis was performed to address concerns about the use of growth hormone therapy in obese patients due to the anti-insulin effects of growth hormone and in patients with underlying respiratory impairment, both of which are very common phenotypes in PWS, among other concerns. Across all studies notable outcomes were an increase in fasting plasma glucose, increases in fasting insulin and HOMA values trending towards significance, and around 15% of all participants experienced edema (Sanchez-Ortiga *et al* 2012). One study even showed an increase in left ventricular mass for 61% of its participants and a mild decrease in left ventricle ejection fraction (LVEF) on echocardiography (Marzullo *et al.* 2007). While the meta-analysis did show a decrease in body fat percentage and an increase in LBM for rhGH treated patients these findings were consistent with other studies in patients with simple obesity, not obesity manifested from PWS (Mekala and Tritos 2009). rhGH does not affect hyperphagia or satiety, but BSN272 could based on its inclusion of DLH (see Table 1). BSN272 could reduce body fat in the adult PWS patients without the concerns that growth hormone supplementation presents.

In addition to the concerns of the use of GH in the overweight population (somatropin use is actually contraindicated in severely obese patients and patients with uncontrolled diabetes) there are concerns of an increased risk of mortality with prolonged use of GH. Somatropin has been associated with tumor promoting potential. The French Sante Adulte GH Enfant (SAGHE) study showed an increase in all-cause mortality in children treated with rhGH, due to diseases of the circulatory system and bone tumors, with a standardized mortality ratio of 1.33 (95% CI of 1.08 - 1.64) (EMA 2012).

Additionally, somatropin is associated with progression of scoliosis in pediatric patients per package insert (Genotropin 2014). Comorbidity of PWS and scoliosis may be as high as 75% (Laurier *et al.* 2014). An observational study of norditropin with thirty-three PWS patients, listed scoliosis as an adverse event in 19.5% of the population (Meinhardt *et al.* 2013). In an additional study of GH treatment, scoliotic conditions worsened in six of the thirteen PWS patients with scoliosis (Murakami *et al*. 2012). Colmenares *et al*. (2011) found scoliosis present in 27.8% of PWS children at baseline, and a scoliosis rate of 47.2% three years later in a prospective cohort study; whereas, other studies have found little statistical difference in the rate of scoliosis between children receiving GH and those not receiving GH (Nagai *et al*. 2006; de Lind van Wijngaarden *et al.* 2009; Odent *et al.* 2008).

Current Hyperlipidemia Medications

Hyperlipidemia may also be a feature of Prader-Willi syndrome, although both normal and elevated lipid levels have been reported in patients. In the treatment of hyperlipidemia, along with diet, there are numerous available medications to reduce blood lipid concentrations. Statins (pravastatin, rosuvastatin, atorvastatin, and simvastatin) are the most commonly prescribed, which act by inhibiting cholesterol production in the liver. Most circulating cholesterol comes from liver production, rather than diet. In addition, inhibition of cholesterol production in the liver stimulates the increased uptake of cholesterol from the blood into the liver, further reducing serum cholesterol. Bile acid resins (cholestyramine, colestipol and colesevalem) exert their effects by binding bile acids present in the large intestine preventing the reabsorption of the bile acids and the absorption of dietary cholesterol. Prevention of bile acid reabsorption results in an increased production of bile acids in liver via the cytochrome P450-mediated oxidation of cholesterol drawn from the blood reducing serum cholesterol. Ezetimibe (Zetia or Ezetrol) localizes at the brush border of the small intestine, and functions by inhibiting cholesterol absorption from the intestine. Proprotein convertase subtilisin/kexin type 9 (PCSK9) is secreted by hepatocytes and binds to LDL receptors to mediate their uptake into hepatocytes and subsequent degradation. This process is targeted by PCSK9 inhibitors. By inhibiting PCSK9, more LDL receptors are left available to bind and remove LDL particles from the circulation.

Despite the introduction of a number of medications such as statins for the treatment of hyperlipidemia, most patients do not achieve optimal LDL levels and drug side effects remain an issue (Mata et al 2011) . Therefore there is a need for a therapy that is highly effective with minimal adverse effects and based upon the pre-clinical and clinical data BSN272 (trans-polydatin + D-lyxo-hexulose) therapy could be beneficial in the treatment of hyperlipidemia.

Table 7. Currently Available Lipid Lowering Therapies

Group	Drug	Benefits	Possible side effects and cautions
Statins:	Altoprev (lovastatin) Crestor (rosuvastatin) Lescol (fluvastatin) Lipitor (atorvastatin) Mevacor (lovastatin) Pravachol (pravastatin) Zocor (simvastatin)	Decrease LDL and triglycerides; slightly increase HDL	Constipation, nausea, diarrhea, stomach pain, cramps, muscle soreness, pain and weakness; possible interaction with grapefruit juice
Bile acid binding resins:	Colestid (colestipol) Questran (cholestyramine sucrose) Welchol (colesevelam)	Decrease LDL	Constipation, bloating, nausea, gas; may increase triglycerides
Cholesterol absorption inhibitors:	Zetia (ezetimibe)	Decrease LDL; slightly decrease triglycerides; slightly increase HDL	Stomach pain, fatigue, muscle soreness
Combination cholesterol absorption inhibitor and statin:	Vytorin (ezetimibe-simvastatin)	Decrease LDL and triglycerides; increase HDL	Stomach pain, fatigue, gas, constipation, abdominal pain, cramps, muscle soreness, pain and weakness; possible interaction with grapefruit juice
Fibrates:	Lofibra (fenofibrate) Lopid (gemfibrozil) TriCor (fenofibrate)	Decrease triglycerides; increase HDL	Nausea, stomach pain, gallstones
Niacin:	Niaspan (prescription niacin)	Decreases LDL and triglycerides; increase HDL	Facial and neck flushing, nausea, vomiting, diarrhea, gout, high blood sugar, peptic ulcers
Combination statin and niacin:	Advicor (niacin-lovastatin)	Decreases LDL and triglycerides; increases HDL	Facial and neck flushing, dizziness, heart palpitations, shortness of breath, sweating, chills; possible interaction with grapefruit juice
Omega-3 fatty acids:	Lovaza (prescription omega-3 fatty acid supplement)	Decreases triglycerides	Belching, fishy taste, increased infection risk
PCSK9 Inhibitors	Praluent (alirocumab) Repatha (evolocumab)	Decrease LDL	Injection site reactions, nasopharyngitis, neurocognitive events

Mechanisms of Action of BSN272

D-lyxo-hexulose

D-lyxo-hexulose, a ketohexose, offers advantages to other sugars such as sucrose and fructose by having a reduced energy value, low glycemic index, prebiotic activity, and

non-cariogenic properties (Bertelsen 1999). Structurally, D-lyxo-hexulose and fructose are highly similar except for an inversion at C-4 in the optically active center (Normen 2001). Although only having this small structural alteration, D-lyxo-hexulose has greatly reduced absorption in comparison to fructose. Studies in both pigs and rats have shown that only 20-25% of ingested D-lyxo-hexulose is absorbed by the small intestine. The remaining D-lyxo-hexulose is fermented by the microbiota in the large intestine into short chain fatty acids and gaseous products (CO_2, methane, and hydrogen) (Saunder 1999; Laerke 1999). The absorption process of D-lyxo-hexulose, unlike the carrier mediated facilitated diffusion of fructose, appears to be passive. D-lyxo-hexulose does not bind to GLUT2 in the gut. DLH does not interfere with the absorption of low levels of fructose (Sigrist-Nelson and Hopfer, 1974; Tatibouet 2000).

Once absorbed, D-lyxo-hexulose metabolism occurs in the liver using the identical biochemical pathway as fructose. Initially D-lyxo-hexulose is phosphorylated by fructokinase to tagatose-1-phosphate, which is further split by aldolase B to yield D-glyceraldehyde (GA) and dihydroxyacetone phosphate (DHAP). Aldolase B acts on both fructose-1-phosphate and tagatose-1-phosphate, although the cleavage of tagatose-1-phosphate occurs at only about half the rate of that of fructose-1-phosphate (Rognstad 1982). Like fructose-1-phosphate, the increase of tagatose-1-phosphate concentration stimulates glucokinase activity (Agius 1994; Van Schaftingen and Vandercammen 1989) leading to an increased phosphorylation of glucose to glucose-6-phosphate, which further activates glycogen synthase (Seoane et al 1996). Along with fructose-1-phosphate, tagatose-1-phosphate inhibits glycogen phosphorylase preventing glycogen from being broken down into glucose subunits. The regulation of these enzymes enables the increase of glycogen synthesis and the decrease of glycogen utilization having the overall net effect of lowering of serum glucose levels (Gergely 1985; Ercan-Fang 2002).

There have been several other suggestions regarding how D-lyxo-hexulose exerts it effect. Studies have proposed that D-lyxo-hexulose may suppress the degradation and absorption of carbohydrates in the intestine by inhibiting intestinal enzymes connected to the brush border membranes. Intestinal enzyme kinetic studies have shown that D-lyxo-hexulose is able to inhibit maltase activity thereby delaying the digestion of starch, and this may account for the lower total energy intake of subjects consuming D-lyxo-hexulose (Seri 1995). In addition to the effect of glycogen regulation, D-lyxo-hexulose inhibits sucrase activity leading to a reduction of sucrose digestion in the small intestine (Seri 1995).

In humans, D-lyxo-hexulose has been orally administered up to 0.64 g/kg/day and was safely tolerated in the Phase 3 study conducted by our group. D-lyxo-hexulose has been shown to reduce blood glucose levels in the treatment of type 2 diabetes and showed significant reduction of hemoglobin A1c (HbA1c) in diabetic patients.

Some patients experience gastrointestinal intolerance to D-lyxo-hexulose but this has been transient and not associated with severe or persistent morbid conditions. Adverse

events in a Phase 2 study at doses of 0.11-0.32 g/kg/day in 107 patients and a Phase 3 study at a dose of 0.64 g/kg/day in 494 patients with diabetes included diarrhea, flatulence, and abnormal gastrointestinal sounds after chronic dosing.

In the United States, D-lyxo-hexulose has been granted generally recognized as safe (GRAS) status under the intended conditions of use in foods (GRAS 78) (http://www.accessdata.fda.gov) currently permitting an estimated intake of 6.6 g/person/day at the mean, and 14.9 g/person/day at the 90th percentile. On July 23, 2003, the Korean Food & Drug Administration (KFDA) authorized the use of D-lyxo-hexulose in foods. In February 18, 2004, Food Standards Australia New Zealand (FSANZ) issued a favorable final assessment report permitting the use of D-lyxo-hexulose as a novel food ingredient (http://www.foodstandards.gov.au). The Joint FAO/WHO Expert Committee on Food Additives (JECFA) evaluated D-lyxo-hexulose at its 55th, 57th, 61st and 63rd meetings. At the 63rd meeting in June 2004, JECFA stated there is no need to limit the acceptable daily intake (ADI) of D-lyxo-hexulose. JECFA has, therefore, established an ADI of "not specified," the safest category in which JECFA can place a food ingredient. On December 14, 2005, D-lyxo-hexulose was formally approved as a "novel food ingredient" in the European Union (EU) without any restriction on usages (http://www.food.gov.uk) of D-lyxo-hexulose.

Trans-polydatin

There appear to be multiple mechanisms of action by which trans-polydatin exerts its actions. Activities include an antioxidant, free radical-elimination mechanism (Hosada 2013, Wang 2015), activation of protein kinase C (Miao 2011, Miao 2012), suppression of NF-kappaB (Fresco 2006), inhibition of the activation of renin-angiotensin-aldosterone system and decreasing the excretion of endothelin 1, TNF-α, and angiotensin II (Zhang 2014), reduction of lipid peroxidation levels (Fabris 2008, Fresco 2006), up regulation of the expression of hippocampal brain-derived neurotrophic factor (Sun 2014), enhanced insulin sensitivity in the liver as shown by improved insulin receptor substrate 2 expression levels and Akt phosphorylation (Hao 2014), decreasing the content of malonydialdehyde (MDA) (Chen 2015), promoting the activities of total superoxide dismutase (T-SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) in plasma, and increasing the content of glutathione (GSH) in myocardial tissue (Wang 2015), restoring decreased deacetylase sirtuin1 activity and protein expression in liver tissue following severe shock (Li 2015) and activation of sirtuin (Huang 2015, Zeng 2015), suppressing oxidative stress-induced lysosomal instability and mitochondrial injury by increasing the protein expression of SOD2 (Li 2015).

The use of polydatin as a potential therapy for dyslipidemia has been suggested primarily by three studies using animal models (Arichi 1982, Du 2009, Xing 2009). Arichi et al (1982) 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 (Du 2009). In another study using rabbits, the administration of polydatin decreased the serum levels of total cholesterol, triglycerides and LDL (Xing 2009). 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 (Whiteman 2002). 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 (Hao 2014). 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 (Xie 2012). Additionally, polydatin is also thought to provide protection from oxidative peroxidation which can result in cell damage (Fabris 2008, Wen 2012) and inhibition of oxidation of LDLs which may also play a role in atherosclerosis (Liu 2012).

Safety of BSN272

D-lyxo-hexulose

D-lyxo-hexulose is Safe for Oral Ingestion by Humans:

In addition to in vitro and animal studies, a number of human studies were conducted in order to support the regulatory approvals for the use of D-lyxo-hexulose in foods. Detailed description of these studies has been made available to the US FDA (GRN 78). The estimated daily intake (EDI) of D-lyxo-hexulose under the intended conditions of use in foods is 6.6 g/person/day (g/p/d) at the mean and 14.9 g/p/d at the 90th percentile. The US FDA raised no questions regarding the conclusion that D-lyxo-hexulose is GRAS under the intended conditions of use. Although the EDI of D-lyxo-hexulose in foods under the intended conditions of use is 6.6 g/p/d, study results supported the safety of D-lyxo-hexulose when it is administered at a dosage of 15 g, three times per day (tid), which is the maximum dosage proposed for this Phase 3 clinical trial. This is supported by the approval of the use of D-lyxo-hexulose in foods by Australia, New Zealand, and the EU with no restriction on usages.

Single-dose and repeated-dose studies in healthy and diabetic human subjects showed that the predominant adverse effects associated with excessive consumption of D-lyxo-

hexulose were gastrointestinal disturbances attributed to osmotic effects from incompletely absorbed D-lyxo-hexulose. At single doses of up to 25 g D-lyxo-hexulose per meal, flatulence was generally the only side effect, with nausea, borborygmi (i.e., rumbling or gurgling noises in the gut), colic, and laxation noted at higher doses (Donner et al., 1996, 1999, 2010; Buemann et al., 1998, 1999a & b, 2000b; Lee and Storey, 1999; Saunders et al., 1999a). Such effects are also commonly associated with excessive consumption of other poorly digestible carbohydrates including polyols. Liver enlargement and elevated uric acid concentration were two concerns during the safety evaluation of D-lyxo-hexulose by the GRAS Expert Panel in the US (D-lyxo-hexulose is self-affirmed as GRAS by a panel of independent experts). Standard toxicity tests with high levels of D-lyxo-hexulose (i.e., diets of 10 to 20% D-lyxo-hexulose) showed a reversible enlargement of the liver in Sprague-Dawley rats without increase in liver enzymes (Bar et al., 1999; Kruger et al., 1999c). The studies were reviewed by Dr. Bar who concluded that the observed liver enlargement in D-lyxo-hexulose fed rats had no relevance to the assessment of human safety of D-lyxo-hexulose (Bar, 1999). However, a clinical trial in humans was important to study the potential effects of D-lyxo-hexulose on the volume of the liver and postprandial liver glycogen concentration. Twelve healthy male subjects were studied in a double-blind crossover study with the ingestion of D-lyxo-hexulose (3 x 15 g daily) and placebo (sucrose, 3 x 15 g daily) for a period of 28 days each. Liver volume and glycogen concentration were determined by magnetic resonance (MR) imaging and spectroscopy. MR examinations before and after the treatments revealed no effects of treatment on liver volume or glycogen concentration. Steady increases in liver volumes, independent of the D-lyxo-hexulose or placebo intake, were observed over the study. The treatment with D-lyxo-hexulose was not associated with clinically relevant changes of the examined clinico-chemical and hematological parameters, including liver enzymes and uric acid (Boesch et al., 2001).

The presence of chronically elevated plasma uric acid levels (i.e., hyperuricemia) is one known risk factor for the development of gout, which is a group of disorders of purine metabolism. The ingestion of single high bolus doses of D-lyxo-hexulose (≥30 g) is associated with a mild, transient increase of plasma uric acid concentration in both healthy subjects and patients with type-2 diabetes (Buemann et al., 2000c; Saunders et al., 1999a; Diamantis and Bar, 2001). However, repeated daily doses of 3 x 15 g D-lyxo-hexulose ingested with the main meals for a period of 28 days produced no effect on fasting plasma uric acid levels in 12 healthy volunteers (Boesch et al., 2001). Another clinical trial in both normal subjects and diabetic patients with the ingestion of D-lyxo-hexulose (3 x 25 g daily) for 8 weeks did not show an increase in fasting plasma uric acid (Saunders et al., 1999a). A pilot study in 8 patients with type-2 diabetes confirmed the non-effect of D-lyxo-hexulose on fasting plasma uric acid at a dosage of 3 x 15 g daily taken with meals for a period of one year (Donner et al., 2010). Furthermore, a clinical trial on 12 hyperuricemic and gouty subjects showed that 15 g D-lyxo-hexulose had no effect of uricemic potential (Diamantis and Bar, 2002). Thus, it is concluded that there is no reason to expect a postprandial increase of plasma uric acid concentrations in response to the ingestion of single 15 g dose of D-lyxo-hexulose in healthy, diabetic,

hyperuricemic, or gouty individuals. Also, it was concluded in the above studies that there were no cumulative effects on fasting serum uric acid levels after chronic intake of D-lyxo-hexulose (i.e., 75 g/day for 8 weeks, or 45 g/day for 12 months).

Possible Adverse Events From D-lyxo-hexulose

In a phase II trial in which D-lyxo-hexulose was administered at 2.5 g, 5.0 g and 7.5 g doses with no run in period, the main reason for failure to complete the trial was withdrawal (37%), subjects asked to leave or were lost to follow up, and not due to an adverse event (AE). The withdraw due to AE's from 2.5 g, 5.0 g and 7.5 g D-lyxo-hexulose was 8.8%, 3.9% and 1.9% respectively. AE's occurrence was found to decrease with increasing D-lyxo-hexulose dosage. The three most prevalent AE's reported were GI disorders, infections, and metabolic and nutritional disorders (see Table 8). Data from a phase III placebo vs. 15.0 g D-lyxo-hexulose trial that contained an 8-week run in period had a withdrawal rate due to AE occurrence of 2.0% and 4.6% respectively. The incidence of infections, metabolic, and nutritional disorders were highly similar in both placebo and D-lyxo-hexulose groups, indicating these occurrences were not study drug related. There was however a 21.5% increased incidence of GI disorders in the D-lyxo-hexulose group in comparison with the placebo (79.5% vs. 58.0%). Note: Predominant populations for the Phase II and III were from India.

Table 8. D-lyxo-hexulose Adverse Effects

	D-lyxo-hexulose				
	Phase II (no run in)			**Phase III (8 wk Run in)**	
Adverse Event (AE)	**2.5 g**	**5.0 g**	**7.5 g**	**placebo**	**15 g**
Total % of AE documented for study	8.80%	3.90%	1.90%	2.00%	4.60%
% of AE that were **gastrointestinal disorders**	44.20%	34.80%	36.2	58.00%	79.50%
% of AE that were **infections and infestations**	19.20%	15.20%	17.00%	23.20%	22.70%
% of AE that were **metabolic & nutritional disorders**	3.80%	8.70%	10.60%	16.40%	16.80%

Trans-polydatin

Possible Adverse Effects of Trans-polydatin

Adverse effects of trans-polydatin in human trials are few and minor in nature, mostly gastrointestinal (Table 7) (Indraccolo et al 2010; Murina et al 2013). In a small pilot study on the effects of phenylethylamine (PEA) and trans-polydatin in patients with endometriosis, one patients reported spotting which the investigators were unable to determine if the spotting was related to either of the investigative therapies (Indraccolo et al 2010).

Table 9. Trans-polydatin Adverse Effects

	trans-polydatin			
	Pilot Study (n=61)[1]	Pilot Study (n=4)[2]	Pilot Study (n=20)[3]	
Adverse Event (AE)	400 mg PEA + 40 mg Trans-polydatin bid	400 mg PEA + 40 mg Trans-polydatin bid	400 mg PEA + 40 mg Trans-polydatin bid	Placebo
Total % of AE documented for study	0	50%	20%	10%
% of AE that were **gastrointestinal disorders**		50%	100%	100%
% of AE that were **gynecological disorders**		50%		

Cobellis et al 2011[1]; Indraccolo et al 2010[2]; Murina et al 2013[3]

In this Phase 1 pharmacokinetic study, the dose of D-lyxo-hexulose would be far less than the dose used in the Phase 3 clinical trial and the dose of trans-polydatin would be 6 orders of magnitude lower than the NOAEL established in the 28-day toxicology study.

Objective

The proposed indication for BSN272 is as a combination therapy for the treatment of patients with Prader-Willi Syndrome.

Primary objective

Determine the pharmacokinetics of a single dose of trans-polydatin in combination with D-lyxo-hexulose. Specifically to test the hypothesis that the t_{max} and c_{max} of a single dose

of trans-polydatin (100 μg) do not change when administered with a single dose of D-lyxo-hexulose (0.03 g/kg).

Subject Population

Healthy male and female volunteers, aged between 18 and 30 years old will be recruited by advertisement at the University of Kentucky. Acceptance into the trial will be based upon the full inclusion and exclusion criteria listed below. After giving informed consent, initial screening performed (physical exam, blood analysis including HIV and Hep C, and urinalysis) and when acceptability of the subject has been determined they will be invited to participate in the study. Subjects will be compensated for their time.

Inclusion Criteria

1. Healthy volunteers (male and female between 18-30 years old).
2. Volunteers weight ≥ 50kg or ≤ 100kg (BMI ≤ 18.5 or ≥ 26)
3. Weight stable (± 10%) for the 3 months prior to starting the study.
4. No history of excessive alcohol consumption.
5. No renal, hepatic, hematological, cardiac or other medical abnormalities detected by basic health check, blood screening by urine test.
6. The subjects must be given signed informed consent before beginning any trial related activities.
7. Individuals have normal dietary habits without restrictions on foods.
8. Negative for HepC, HIV, and pregnancy.

Exclusion Criteria

1. Any unexpected weight loss (-10%) during the last 3 months.
2. Volunteers weight ≤ 50 kg or ≥ 100 kg (BMI ≤ 18.5 or ≥ 26)
3. Known or suspected illicit drug or alcohol abuse.
4. Known Impaired hepatic or renal functions.
5. Undergone any gastrointestinally-related surgeries.
6. Any hematological or cardiac abnormalities.
7. Uncontrolled hypertension (systolic pressure >180 mm Hg and/or diastolic pressure >100 mm Hg).
8. Recent or ongoing major disease or cancer.
9. Has gastroesophageal reflux disease (GERD)
10. Has irritable bowel disease (IBS)
11. Has inflammatory bowel disease (IBD)
12. Any or recent gastric bleeding.
13. Current gastric or duodenal ulcers.
14. History of esophageal motility disorders.

15. Concomitant usage of daily nonsteroidal anti-inflammatory drugs (aspirin > 325 mg/day).
16. Oral corticosteroids (prednisone and prednisolone).
17. Anticholinergics. ((Benztropine (Cogentin), Ipratropium (Atrovent), Oxitropium (Oxivent), Diphenhydramine (Benadryl, Sominex, Equate Sleep Aid, Advil PM, etc.), Dimenhydrinate (Dramamine)).
18. Usage of therapeutic antacids or H2 receptor antagonists.
19. Taking enzyme therapy within the last 14 days, proton pump inhibitor within the last 7 days, or octreotide within 48 hours.
20. Any known hypersensitivity to formulation ingredients.
21. Subject history of viral, bacterial, or fungal infection within 4 weeks of screening
22. Receiving any investigational drug within 30 days of the screening visit.
23. Any behavioral or social problems that may interfere with the clinical study procedures.
24. Patient is unlikely, in the investigator's opinion, to complete the study
25. Have consumed grapes, grape-containing products, peanuts, peanut-containing products, blueberries, blueberry-containing products, chocolate, and chocolate-containing products, probiotics, prebiotics, and nutritional supplements in the last **2 days.**
26. Pregnancy, breastfeeding, or intention of becoming pregnant or judged to be using inadequate contraceptive measure.

Subject Withdrawal

Subjects have the right to withdraw from the study at any time for any reason without prejudice. The investigator also has the right to withdraw subjects from the study in the event of illness, adverse events, protocol deviations, administrative, and other reasons. The subject should, if possible, remain in the study (in the absence of treatment with study material) for the collection of safety parameters at the end of the trial.

It is understood by all concerned that an excessive rate of withdrawals can render the study statistically underpowered for interpretation. Therefore, unnecessary withdrawal of subjects should be avoided. Should a subject decide to withdraw, all efforts will be made to complete and report the observations as thoroughly as possible and collect study products. If a subject withdraws from the study due to an adverse event, the specific event will be recorded on the Adverse Event Case Report Form (CRF) and the subject will be followed until the event is resolved.

Subject Instruction

Volunteers will receive, read, and sign a copy of the informed consent form before any trial related activities are undertaken. The consent form will outline all study appointments, health screening process, procedures requirements involved at each appointment.

Concomitant Interventions

As the trial is being conducted in healthy volunteers they will be prohibited from taking other medications

Study Design

The main objective of this phase 1 clinical trial is to test the hypothesis that administration of trans-polydatin with D-lyxo-hexulose does not change the t_{max} or c_{max} of trans-polydatin. All volunteers will be healthy male and female aged 18-30 years old, must not be taking other medications, prescription or nonprescription, with no excessive alcohol intake, and have not previously participated in a clinical trial within the last 30 days. Those potentially eligible subjects meeting the initial inclusion and exclusion requirements, and after signing informed consent, will undergo a basic physical examination, hematology, blood chemistry, and urinalysis. This screening and recruitment process should be complete within 3-5 days. After meeting all trial criteria, eligible subjects are invited to participate in the trial. They will be asked to attend the trial facility and will be randomly assigned to one of the two treatment groups for the duration of the study. Randomization is to be stratified according to sex to achieve a balanced distribution of subjects across the 2-treatment groups. The treatment groups will be (1) trans-polydatin and (2) BSN272 (trans-polydatin + D-lyxo-hexulose). Six subjects, 3 male and 3 female, will be assigned to each group for a total of 12 subjects. On the trial day subjects will be given study medication and then timed blood samples will be taken for pharmacokinetic analysis. Urine samples will also be collected during the 12 hours the volunteers are at the CCTS, with one subsequent urine collection at visit 3, which should approximate 24 hours post-treatment, and will be stored for contingent analysis. Serum and urine trans-polydatin and trans-resveratrol will be determined.

For this study, potential subjects will be screened to identify 16 study healthy volunteers (8 male, 8 female) of which 12 participants (6 male, 6 female) will be used for the actual study with 4 alternates (2 male, 2 female) in case a participant cannot complete the study. Six subjects, 3 male and 3 female, will be assigned to each group.

At the initial recruitment/screening a full explanation of the trial requirements and expectations will be given to the volunteers and a signed consent will be obtained before beginning the screening process. Then every consenting volunteer will provide medical history, have their vital measurements taken (weight, height, and BP), undergo a basic physical examination, blood samples taken for hematology (hematocrit, hemoglobin, MCH, MCHC, MCV, total WBC, platelet count, and differential), clinical chemistry (sodium, potassium, chloride, CO_2, BUN, albumin, creatinine clearance, SGOT, ALT, glucose, total bilirubin, HIV, and Hep C) and urinalysis (appearance, pH specific

gravity, glucose, protein, microscopic evaluation of urinary sediment). When all of the data from these screening tests have been analyzed and the eligibility of the volunteers determined, they will be asked to participate and scheduled to return for the study.

On the first day of the study all the enrolled subjects will attend the study clinic where they will be administered the trial drug and have timed blood samples taken. They will be required to have fasted for 12 hours prior to attending the trial facility at the start of each trial arm. On the first trial day volunteers will be randomly assigned into 2 groups (trans-polydatin or BSN272). Once assigned to a treatment group volunteers will remain in that group for the duration of the study. Each subject will then have blood tests taken for baseline trans-polydatin and trans-resveratrol levels.

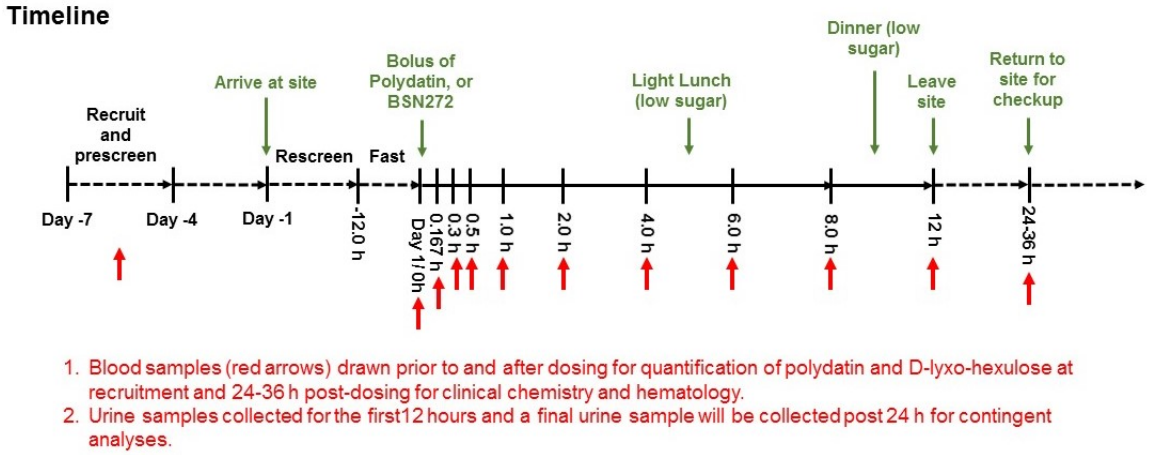
Each subject will drink the study drug(s) assigned to their group dissolved in volume of approximately 4 ounces of water per drug. Each drug will be dissolved separately in water and subjects will drink both solutions (trans-polydatin in the trans-polydatin group, or trans-polydatin and D-lyxo-hexulose in the BSN272 group) from separate cups in rapid succession. The study dosage is shown in Table 10.

Table 10. Study Drug Dosage Schedule

Group (Arm)	Treatment	
	Cup #1	**Cup #2**
Trans-polydatin (n = 6; 3 male, 3 female)	Trans-polydatin (100 µg)	Water
BSN272 (n = 6; 3 male, 3 female)	Trans-polydatin (100 µg)	D-lyxo-hexulose (0.03 g/kg)

After administration of the study drugs, blood samples will then be taken at approximately 0.167 (10 mins), 0.333 (20 mins), 0.5, 1, 2, 4, 6, 8, 12 and once between 24-36 hours post dose. Urine samples will also be collected during the 12 clinical period, with one subsequent collection at the 24-hour time point (visit 3). Subjects will be required to attend the trial facility after a 12 hours overnight fast. Subjects will remain in the facility, and monitored constantly for safety for the duration of the timed pharmacokinetic blood collection (12 hours). The subjects will receive a light, low polydatin lunch after the 4 hour blood sample has been taken. After the 12-hour sample draw they will be allowed to leave but will be required to return for the following day for the 24-hour sample collection.

Figure 1. Trial Timeline Overview



1. Blood samples (red arrows) drawn prior to and after dosing for quantification of polydatin and D-lyxo-hexulose at recruitment and 24-36 h post-dosing for clinical chemistry and hematology.
2. Urine samples collected for the first12 hours and a final urine sample will be collected post 24 h for contingent analyses.

Clinical and Laboratory Evaluations

The trial evaluations are illustrated in Table 11. At visit 1 (screening) the volunteer's medical history, medications, physical assessment, and laboratory evaluation will be performed. Laboratory tests include electrolytes (sodium, potassium, chloride, and bicarbonate), renal function (BUN and creatinine), Liver function (AST, ALT, CK) albumin, alkaline phosphatase, total bilirubin and LDH at baseline and then at the end of the trial. The study into the pharmacokinetic effects of increasing BSN272 therapy will be performed over 2 arms of this trial. Blood samples will be taken at the time points 0, 0.167, 0.333, 0.5, 1, 2, 4, 6, 8, 12 and 24-36 hours post study drug, and a 24 hour urine will be collected during the trial. Trans-polydatin and trans-resveratrol will be measured in both the plasma and urine samples. The volunteers' health will be closely monitored during the trial and any unexpected or adverse effects recorded. Any adverse results found during the initial screening during visit 1 the volunteer will be advised and omitted from proceeding with the clinical trial.

The clinical samples taken during the screening process and at the end of the trial will be analyzed at the University of Kentucky clinical chemistry laboratory. A list of laboratory tests is summarized in Appendix I. The analysis of trans-polydatin and trans-resveratrol levels will be performed in-lab at the University of Kentucky.

Table 11. BSN272 Trial Evaluations

Procedure	Screening	Trial arms 1 and 2.											Final Day of Arm 2
		Time post study drug.											Additional tests
		0	0.167	0.333	0.5	1.0	2.0	4.0	6.0	8.0	12.0	24.0	24hr
Registration	X												
Entry criteria	X												
Informed Consent	X												
Blood work	X												X
Vitals-weight, BMI, height	X												X
Administer Study drugs		X											
Medical History	X												
Arrive after 12 hour fast		X											
Record Adverse Events		X	X	X	X	X	X	X	X	X	X	X	
PK blood sample collection for trans-resveratol and D-tagatose levels	X	X	X	X	X	X	X	X	X	X	X	X	
24 hour Urine collection.												X	
Compensation.	X											X	

Clinical Supplies

Study Supplies and Labeling

We will supply the following

- trans-polydatin
- D-lyxo-hexulose

The study material (trans-polydatin and D-lyxo-hexulose) will be supplied directly to the study clinic. Each subject will be supplied their own individual doses in water with a specific amount trial drug as described in Table 10. Each dose will be clearly labeled with its contents and the individual subject's trial number.

Accountability

All materials supplied are for use only in this clinical trial and will not be used for any other purposes. The investigators or designees will maintain a full record of product accountability. A study dispensing log will be kept current and contain the following information:

- The identification of subject to whom the product is dispensed, using volunteer initials and number.
- The date and code of product dispensed for treatment for each volunteer.

The above inventory will be available for inspection by an un-blinded study monitor that is not responsible for monitoring of the case report forms.

Trial Drug Storage

The study products (D-lyxo-hexulose and trans-polydatin) will be stored at a temperature of 15 to 25 degrees C or 59 to 77 degrees F. Trans-polydatin is light sensitive and will be stored in light-tight, opaque containers.

Visit Schedule

Visit 1 (Recruiting and Screening)

Healthy volunteers are eligible for screening for acceptance into this clinical trial. Prior to screening and enrollment each volunteer will be provided both verbal and written study relevant information and will have any questions or concerns they have answered. If the volunteers make the decision to participate in the trial, they will then complete and sign an informed consent form. At this point, based upon the inclusion and exclusion criteria the potential volunteer's medical history will be recorded, have initial blood samples taken and be given a basic physical exam. The basic physical exam includes physical measurements (weight, height and BMI), general examination by observation (inspection), blood pressure, and heart rate check. The clinical laboratory tests include (1) hematology (hematocrit, hemoglobin, MCH, MCHC, MCV, total white blood cells, platelets, and differential), and (2) clinical chemistry (sodium, chloride, potassium, CO_2, BUN, uric acid, albumin, creatinine clearance, SGOT, ALT, bilirubin, phosphorus, calcium, alkaline phosphatase, total protein, and glucose. Also HIV and Hep C screening will be performed. Urine analysis will also be performed that includes appearance, volume, specific gravity, pH, glucose, protein, and microscopic evaluation of urinary sediment. Any samples that have a hematological, biochemical or physical component that is outside normal ranges, or a positive HIV or Hep. C result is obtained the volunteer will be informed but omitted from proceeding with the study.

All subjects who are eligible to be enrolled in the study will be informed by a phone call to schedule a follow-up appointment approximately 1 week from the screening visit. Enrolled volunteers will be randomized (stratified across all groups according to sex into the study). Each volunteer will be assigned a volunteer number. The volunteer's number and the volunteer's three initials (unless they only have two initials) will be the identifiers recorded on the CRFs.

Visit 2 (Trial Initiation)

For 12 hours prior to attending the trial facility the volunteers will be required to fast. No food to be consumed from 10 pm the night prior to the trial. On day 1 of the trial, volunteers will be fitted with an angiocath for ease of timed pharmacokinetic blood sample collection. At this point a blood sample will be taken for baseline analysis of

trans-polydatin, and trans-resveratrol. Each subject will then be given the study drug(s) that has been individually prepared for them based upon the trial group and the individual's body weight (Table 12).

Table 12. Trial Dosages

Group	Trans-polydatin (μg)	D-lyxo-hexulose (g/kg)
Trans-polydatin	100	-
BSN272	100	0.03

Pharmacokinetic blood samples will be taken at the time points 0, 0.167, 0.333, 0.5, 1, 2, 4, 6, 8, 12 and once between 24-36 hours post study drug administration. Urine samples will be collected during the 12 hours post-treatment period with a subsequent final urine sample to be collected during visit 3. Blood samples will be collected in heparinized tubes and will be kept on ice until plasma separation by centrifugation. During blood collection time intervals the subject will remain in the clinical test facility and will be monitored constantly for any adverse reactions. After the 12 hour sample has been drawn the volunteer will have the angiocath removed and be discharged. They will be asked to return the following morning for the 24 hour sample time point. After the 4 hour blood sample collection all volunteers will be given a light, low sugar lunch.

Visit 3 (24 Hour Follow-Up)

All volunteers will return to the trial facility to have the 24-hour time point blood sample drawn and a final urine collection.

Determination of Sample Size

Potential subjects will be screened to identify 16 study healthy volunteers (8 male, 8 female) of which 12 participants (6 male, 6 female) will be used for the actual study. There will be 2 treatment groups each having 6 volunteers randomly assigned. The determination of the minimum study group numbers is based upon a 28 day rat toxicology study taken at 1-hour post trans-polydatin, 2-sided, confidence interval of, (alpha 0.025) and a power of 90%.

Statistical Analysis

The primary objective of this phase 1 microdose study is to determine whether the t_{max} and C_{max} of t-PD change significantly when coadministered with DLH in healthy male and female volunteers.

Non-compartmental analysis will be applied to the mean plasma trans-polydatin and trans-resveratrol concentration data from the volunteers. The following parameters will be estimated whenever possible:

1. C_{max} - Maximum observed concentration
2. T_{max} - Time to maximum observed concentration.

C_{max} and T_{max} will be determined by visual inspection of the concentration-time data. If any maxima appear identical, a computer sort will identify the highest data value.

AUC_{0-8} - Area under the concentration-time curve from hour 0 to hour 8, estimated by the linear trapezoidal rule.
AUC_{0-t} - Area under the concentration-time curve from hour 0 to the last measurable concentration, estimated by the linear trapezoidal rule.

$AUC_{0-\infty}$ - Area under the concentration-time curve from hour 0 to infinity for Day 1 calculated as follows:
$AUC_{0-\infty} = AUC_{0-t} + C_t/\lambda z$

Where C_t is the last measurable concentration and λz is the elimination rate constant estimated using log-linear regression during the terminal elimination phase. The number of points used in λz calculation will be determined by visual inspection of the data describing the terminal phase. At least the last three time points with measurable values will be used in λz calculation.

M/P ratio - Metabolite to Parent ratio calculated as: AUC_{0-8} metabolite/AUC_{0-8} parent pharmacokinetic analysis will be performed using WinNonlin Professional Edition (Pharsight Corporation, Version 5.2). Nominal doses and sampling times will be used. Dose normalized parameters, ratios, and descriptive statistics will be calculated using WinNonlin and Microsoft Excel (Version 11.0). Concentration values found below the lower limit of quantification (< 20.0 ng/mL for trans-polydatin and < 10.0 ng/mL for trans-resveratrol) will be treated as zero for descriptive statistics and toxicokinetic analysis.

A t test (p=0.05) will be used to calculate the significance of the difference between the C_{max} and T_{max} of t-PD with and without DLH.

Assay

In an earlier validation study for a of rat plasma PK study, the validated range of trans-polydatin was 20 to 20000 ng/mL (Biospherics Study No. 70971-0010). It is expected to be similar for human plasma levels but the UK Clinical laboratory's analytical method for supported-liquid extraction / LC-MS/MS of trans-polydatin will require validation.

Study Sample Handling

Study samples will be transported by CCTS personnel to the Kentucky Clinic Laboratory. Samples will be stored in a freezer set to maintain -60 to -80 ^{0}C.

Quality Control

A small dilution concentration quality control sample of the prepared BSN 272 will be collected at the zero time point, to assess dilution QC. Results will be reported to study sponsor.

Each batch of samples analyzed should include a calibration curve, a matrix blank, a control zero (matrix blank containing internal standard), a reagent blank, and duplicate quality control (QC) samples at three concentrations within the calibration range. The samples were interspersed with calibration standards and QC samples within the batch.

Sample results will be considered valid if calibration curve and QC data indicate the method met the acceptance criteria, which includes: no more than 1/4 of the standards were excluded, 1/2 of the undiluted QC samples and 2/3 undiluted QC samples are within the 85-115% range, and at least 2/3 of the original results are within 20% of each other.

Safety Outcomes

Adverse Events Monitoring

Definitions:

An adverse event (AE) is any untoward medical occurrence in a patient, in a clinical investigation administered a pharmaceutical product, which does not necessarily have to have a causal relationship with this treatment.

An AE is therefore any unfavorable and unintended sign (including an abnormal laboratory finding), symptom or disease temporally associated with the administration of an investigational product, whether or not related to that investigational product. An unexpected AE is one of a type not identified in nature, severity, or frequency in the current Investigator's Brochure or of greater severity or frequency than expected based on the information in the Investigator's Brochure.

A Serious Adverse Event is any untoward medical occurrence at any dose that:
Results in death or;

- Is life-threatening or;

Note: The term "life-threatening" in the definition of "serious" refers to an event in which the patient was at risk of death at the time of the event; it does not refer to an event that hypothetically might have caused death if it were more severe.

· Requires inpatient hospitalization or prolongation of existing hospitalization or;
· Results in persistent or significant disability/incapacity or; Is a congenital anomaly/birth defect;
· Is a medically important event:

Medical and scientific judgment should be exercised in deciding whether expedited reporting is appropriate in other situations, such as important medical events that may not be immediately life-threatening or result in death or hospitalization but may jeopardize the patient or may require intervention to prevent one of the other outcomes listed in the definition above. These should also usually be considered serious.

The Common Terminology Criteria for Adverse Events (CTCAE) Version 4.0 should be used to assess and grade AE severity, including laboratory abnormalities judged to be clinically significant. The modified criteria can be found in the study manual. If the experience is not covered in the modified criteria, the guidelines shown in Table 13 below should be used to grade severity. It should be pointed out that the term “severe” is a measure of intensity and that a severe AE is not necessarily serious.

Table 13. AE Severity Grading

Severity (Toxicity Grade)	Description
Mild (Grade 1)	Mild; asymptomatic or mild symptoms; clinical or diagnostic observations only; intervention not indicated.
Moderate (Grade 2)	Moderate; minimal, local or noninvasive intervention indicated; limiting age-appropriate instrumental ADL*.
Severe (Grade 3)	Severe or medically significant but not immediately life-threatening; hospitalization or prolongation of hospitalization indicated; disabling; limiting self-care ADL**.
Life-threatening (Grade 4)	Life-threatening consequences; urgent intervention indicated.

Activities of Daily Living (ADL)
*Instrumental ADL refer to preparing meals, shopping for groceries or clothes, using the telephone, managing money, etc.
**Self care ADL refer to bathing, dressing and undressing, feeding self, using the toilet, taking medications, and not bedridden.

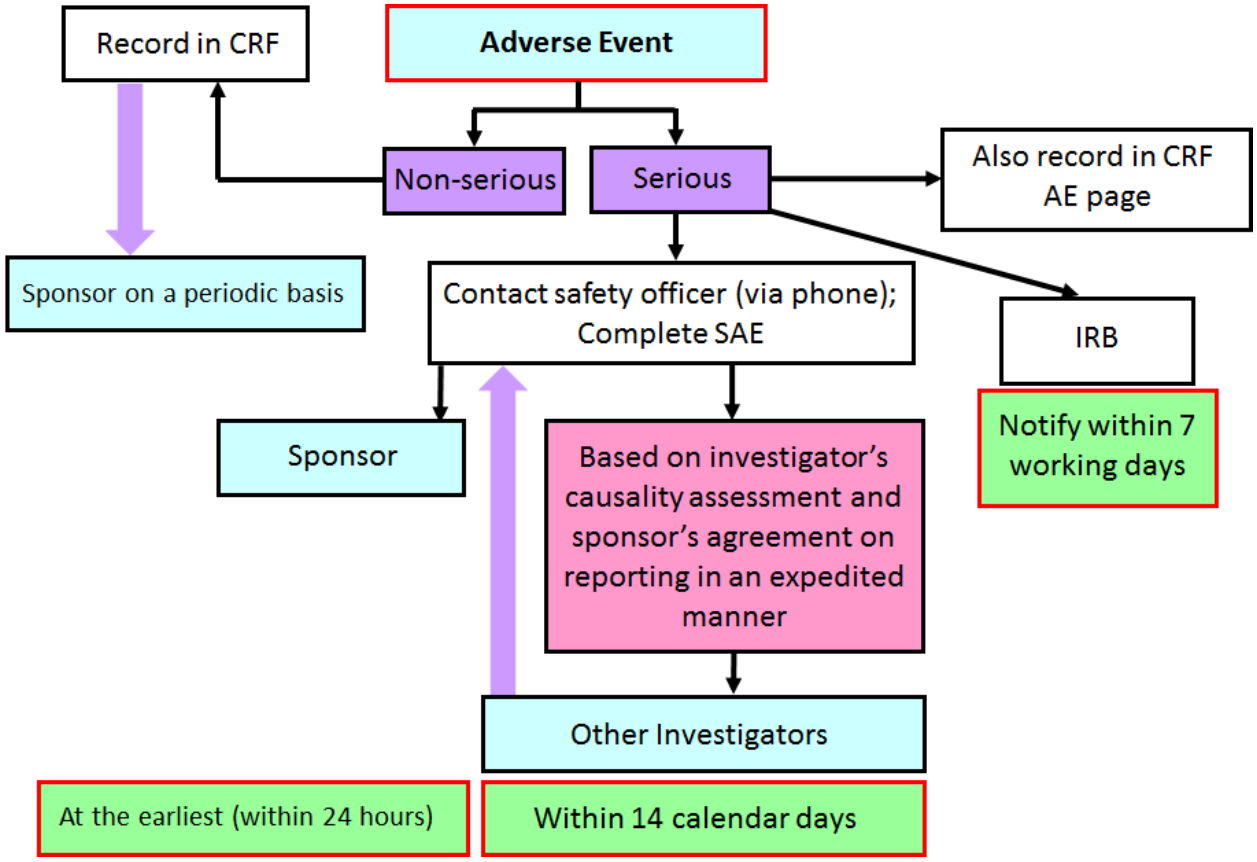
AE Relationship to Study Drug

The relationship of an AE to the study drug should be assessed using the following the guidelines in Table 14.

Table 14. AE Relationship to Study Drug

Relationship to Drug	Comment
Definitely	Previously known toxicity of agent; or an event that follows a reasonable temporal sequence from administration of the drug; that follows a known or expected response pattern to the suspected drug; that is confirmed by stopping or reducing the dosage of the drug; and that is not explained by any other reasonable hypothesis.
Probably	An event that follows a reasonable temporal sequence from administration of the drug; that follows a known or expected response pattern to the suspected drug; that is confirmed by stopping or reducing the dosage of the drug; and that is unlikely to be explained by the known characteristics of the subject's clinical state or by other interventions.
Possibly	An event that follows a reasonable temporal sequence from administration of the drug; that follows a known or expected response pattern to that suspected drug; but that could readily have been produced by a number of other factors.
Unrelated	An event that can be determined with certainty to have no relationship to the study drug.

Adverse Events Reporting



Responsible Parties

Sponsor/Investigator/ Safety Officer- Robert Lodder
Other Investigators - Medical Investigators (site PIs)
The Sponsor-Investigator/Safety Officer will inform the IRB of any events of which he becomes aware.

Serious Adverse Event Reporting

Safety reports will be made of potential serious risks, from clinical trials or any other source, as soon as possible, but in no case later than 14 calendar days after the sponsor determines that the information qualifies for reporting.

The PI will report any suspected adverse reaction that is both serious and unexpected. The sponsor must report an adverse event as a suspected adverse reaction only if there is evidence to suggest a causal relationship between the therapeutic agent and the adverse event, such as:

(A) A single occurrence of an event that is uncommon and known to be strongly associated with drug exposure (e.g., angioedema, hepatic injury, Stevens-Johnson Syndrome);

(B) One or more occurrences of an event that is not commonly associated with drug exposure, but is otherwise uncommon in the population exposed to the drug (e.g., tendon rupture);
(C) An aggregate analysis of specific events observed in a clinical trial (such as known consequences of the underlying disease or condition under investigation or other events that commonly occur in the study population independent of drug therapy) that indicates those events occur more frequently in the drug treatment group than in a concurrent or historical.

Life-threatening suspected adverse reaction as soon as possible but in no case later than 7 days after sponsor determines that the information.

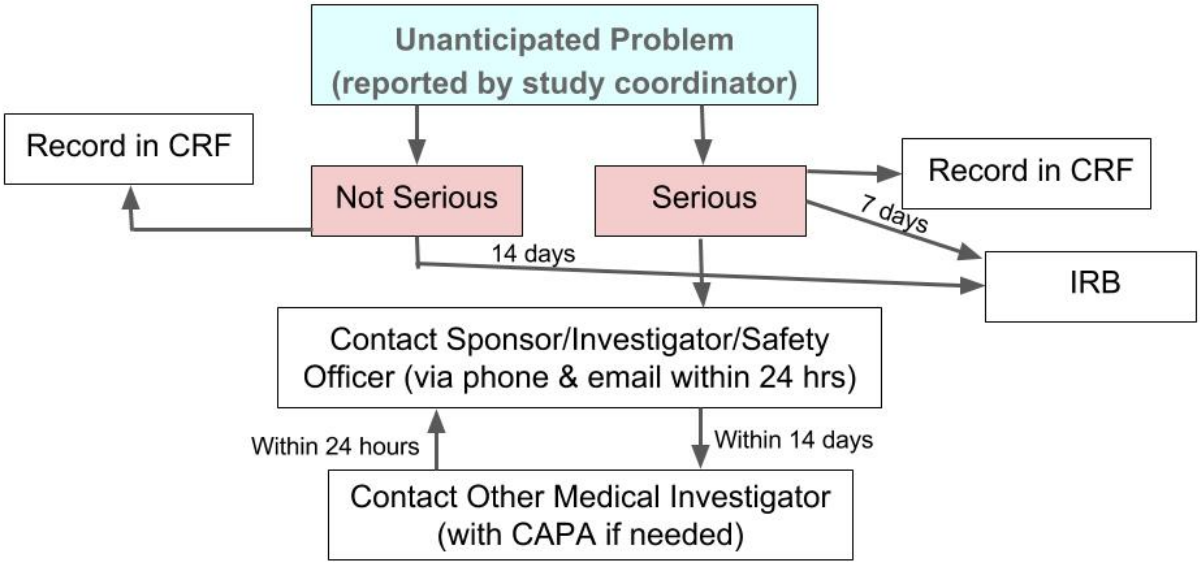
The study clinician will complete a SAE Form within the following timelines:

- All deaths and immediately life-threatening events, whether related or unrelated, will be recorded on the SAE Form and submitted to the study sponsor within 24 hours of site awareness.
- Other SAEs regardless of relationship, will be submitted to the study sponsor within 24 hours of site awareness.

All SAEs will be followed until satisfactory resolution or until the site investigator deems the event to be chronic or the adherence to be stable. Other supporting documentation of the event may be requested by the study sponsor and should be provided as soon as possible.

The study sponsor will be responsible for notifying IRB of any unexpected fatal or life-threatening suspected adverse reaction as soon as possible but in no case later than 7 calendar days after the sponsor's initial receipt of the information.

Unanticipated Problem Reporting



Incidents or events that meet the OHRP criteria for UPs require the creation and completion of an UP report form. It is the site investigator's responsibility to report UPs to their IRB. The UP report will include the following information:

- Protocol identifying information: protocol title and number, PI's name, and the IRB project number;
- A detailed description of the event, incident, experience, or outcome;
- An explanation of the basis for determining that the event, incident, experience, or outcome represents an UP;
- A description of any changes to the protocol or other corrective actions that have been taken or are proposed in response to the UP.

To satisfy the requirement for prompt reporting, UPs will be reported using the following timeline:

- UPs that are SAEs will be reported to the IRB within 7 days of the investigator and to the sponsor within 24 hours of the investigator becoming aware of the event.
- Any other UP will be reported to the IRB and to the study sponsor within 14 days of the investigator becoming aware of the problem.
- All UPs should be reported to appropriate institutional officials (as required by UK per Serious or Continuing Noncompliance or Unanticipated Problems Involving Risks:IRB Reporting to Federal Agencies using the UK IRB reporting form within the timeline in accordance with the SOP for Unanticipated/Anticipated Problem/Adverse Event Reporting (UK) of the IRB's receipt of the report of the problem from the investigator.

List of Regulatory Agency
IRB
Responsible Parties
Sponsor/ Safety Officer - Robert Lodder
Medical investigators (site PIs)
Sponsor/Safety Officer and the IRB

Ethical and Regulatory Standards

Ethical Principles

This Clinical Trial will be conducted in accordance with the principles laid down by the 18th World Medical Assembly (Helsinki, 1964) and all applicable amendments laid down by the World Medical Assemblies, and the ICH guidelines for Good Clinical Practice (GCP). Those Investigators participating as leaders in this trial, including members of the Executive and Operations Committees and National Coordinators, will receive compensation for their time but will receive no financial profit from their activities related to the trial.

Laws and Regulations

This Clinical Trial will be conducted in compliance with all international laws and regulations, and national laws and regulations of the countries in which the clinical trial is performed, as well as any applicable guidelines. The trial will be registered on www.clintrials.gov and on other sites, as appropriate.

Informed Consent

The Investigator (according to applicable regulatory requirements), or a person designated by the Investigator, and under the Investigator's responsibility, should fully inform the patient of all pertinent aspects of the clinical trial, including the written information given approval/favorable opinion by the Institutional Review Board/Ethics Committee Prior to a patient’s participation in the clinical trial, the written Informed Consent Form should be signed, name filled in and personally dated by the patient or by the patient’s legally acceptable representative, and by the person who conducted the informed consent discussion. A copy of the signed and dated written Informed Consent Form will be provided to the patient. If informed consent is obtained under special circumstances (emergency, from a guardian, minor, etc.), the method should be specified following the ICH requirements. The first part of the section should be adapted, keeping the point as appropriate. The Informed Consent Form used by the Investigator for obtaining the patient's informed consent must be reviewed and approved by the Sponsor prior to submission to the appropriate IRB for approval/favorable opinion.

Institutional Review Board (IRB)

The Investigator must submit this Clinical Trial Protocol to the appropriate IRB, and is required to forward to the Sponsor a copy of the written and dated approval/favorable

opinion signed by the Chairman with IRB composition. The Clinical Trial (study number, Clinical Trial Protocol title and version number), the documents reviewed (Clinical Trial Protocol, Informed Consent Form, Investigator's Brochure, Investigator's CV, etc.) and the date of the review should be clearly stated on the written IRB approval/favorable opinion. Investigational Product will not be released at the study site and the Clinical Trial will not start until a copy of this written and dated approval/favorable opinion has been received by the Sponsor. During the Clinical Trial, any amendment or modification to the Clinical Trial Protocol should be submitted to the IRB. It should also be informed of any event likely to affect the safety of patients or the continued conduct of the Clinical Trial, in particular any change in safety. All updates to the Investigator's Brochure will be sent to the IRB. If requested, a progress report is sent to the IRB annually and a summary of the Clinical Trial's outcome at the end of the Clinical Trial.

Study Monitoring

Responsibilities of the Investigator(s)

The Investigator(s) undertake(s) to perform the Clinical Trial in accordance with this Clinical Trial Protocol, ICH guidelines for Good Clinical Practice and the applicable regulatory requirements. The Investigator is required to ensure compliance with all procedures required by the Clinical Trial Protocol and by study procedures provided by the Sponsor (including security rules). The Investigator agrees to provide reliable data and all information requested by the Clinical Trial Protocol (with the help of the Case Report Form [CRF], Discrepancy Resolution Form [DRF] or other appropriate instrument) in an accurate and legible manner according to the instructions provided and to ensure direct access to source documents to Sponsor representatives. If any particular circuits have to be defined (e.g., e-CRF, Fax), particular attention should be paid to the confidentiality of the patient's data to be transferred. The Investigator may appoint such other individuals as he/she may deem appropriate as Sub-Investigators to assist in the conduct of the Clinical Trial in accordance with the Clinical Trial Protocol. All Sub-Investigators shall be timely appointed and listed. The Sub-Investigators will be supervised by and under the responsibility of the Investigator. The Investigator will provide them with a Clinical Trial Protocol and all necessary information.

Responsibilities of the Sponsor

The Sponsor of this Clinical Trial is responsible to Health Authorities for taking all reasonable steps to ensure the proper conduct of the Clinical Trial Protocol as regards ethics, Clinical Trial Protocol compliance, integrity and validity of the data recorded on the Case Report Forms. Thus, the main duty of the Monitoring Team is to help the Investigator and the Sponsor maintain a high level of ethical, scientific, technical and regulatory quality in all aspects of the Clinical Trial. At regular intervals during the Clinical Trial, the site will be contacted, through monitoring visits, letters or telephone calls, by a representative of the Monitoring Team to review study progress, Investigator and patient compliance with Clinical Trial Protocol requirements, and any emergent

problems. During these monitoring visits, the following but not exhaustive list of points will be scrutinized with the Investigator: patient informed consent, patient recruitment and follow-up, Serious Adverse Event documentation and reporting, outcome events documentation and reporting, Investigational Product allocation, Investigational Product accountability, concomitant therapy use and quality of data.

Source Document Requirements

According to the ICH guidelines for Good Clinical Practice, the Monitoring Team must check the Case Report Form entries against the source documents, except for the pre-identified source data directly recorded in the Case Report Form. The Informed Consent Form will include a statement by which the patient allows the Sponsor's duly authorized personnel, the Ethics Committee (IRB), and the regulatory authorities to have direct access to source data which supports the data on the Case Report Forms (e.g., patient's medical file, appointment books, original laboratory records, etc.). These personnel, bound by professional secrecy, must keep confidential all personal identity or personal medical information (according to confidentiality rules).

Use and Completion of Case Report Forms (CRFs) and Additional Requests

It is the responsibility of the Investigator to maintain adequate and accurate CRFs designed by the Sponsor to record all observations and other data pertinent to the clinical investigation. All source documents should be completed in their entirety in electronic format to ensure accurate interpretation of data, which should be entered within 48 hours of completion or any modification into the eCRF. Should a correction be made, the information will be re-entered with tracked changes that show who made which changes and when. The corrected information will be entered by the authorized person into the eCRF. The computerized handling of the data by the Sponsor after receipt of the CRFs may generate additional requests (DRF) to which the Investigator is obliged to respond by confirming or modifying the data questioned. Unresolved requests will be entered at least every 2 weeks.

Study Documentation, Case Report Forms, and Record Keeping

Investigator's File

The investigator must maintain adequate and accurate records to enable the conduct of the study to be fully documented and the study data to be subsequently verified, in electronic format with tracked changes. These documents should be classified as two types: investigators study master file and study/subject clinical source documents.

Source Documentation

Source documents are defined as the primary source for all study related data. They are considered original documents, data, and records. This may include hospital records, clinical and office charts, laboratory data/information, and pharmacy dispensing and

other records. All entries in the CRF must be supported by data recorded on the source documentation.

Source documentation will be kept in the individual subject's file during the conduct of the clinical trial and stored in a secure electronic database. The investigator(s) will permit study-related monitoring, audits, IEC review, and regulatory inspection(s), providing direct access to source documents.

Case Report Forms

All forms will be filled out in electronic format with automatic version control. The study monitor at the study site will review CRFs. Errors detected by subsequent in-house CRF review may necessitate clarification or correction of errors. All changes will be documented and approved by the investigator.

Data Handling

All data will be entered into electronic CRFs with tracked changes. All data manually entered in the database will be verified by a double-key entry procedure. After completion of the entry process, computer logic checks will be run to check for such items as inconsistent study dates and outlying laboratory values. Any necessary corrections will be made to the database and documented via tracked changes. A manual review of selected line listings will also be performed at the end of the study. The data from clinical laboratory will be provided electronically and entered into the CRF by a double-key entry procedure. In the event of conflicts, a third person will meet with the first two to resolve the conflict. A Discrepancy Resolution Form (DRF) will track the process and the outcome.

Protocol Amendments

All amendments must be reviewed and approved by the applicable IRB/ERB(s) in the US before the revised edition can be implemented.

Confidentiality

The investigator will assure that subjects' anonymity will be maintained. On CRFs or other documents submitted to CRO, subjects will not be identified by their names, but by an identification code. The investigator will keep a separate log of subjects' codes and names. Documents not for submission to CRO, such as informed consent forms and medical records, will be kept in strict confidence.

Study Interventions, Administration, and Duration

After initial screening (Visit 1) the volunteers will be randomly divided into two groups. One will be given trans-polydatin, which will be administered as a single oral dose of 100 μg. The second group will receive BSN272 (100 μg trans-polydatin and D-lyxo-hexulose at 0.03 g/kg in separate cups). Blood samples for pharmacokinetic study and toxicology monitoring will be taken at the proposed time points for the following 24 hours. Urine samples will also be collected for the initial 12 hours so that elimination of the trial drug can be monitored, with one subsequent urine sample during visit 3. Post study subjects will be monitored/asked to report and adverse effects. Medical intervention will take place if toxicological symptoms arise.

Appendix 1 - Clinical Laboratory Tests

Serum Chemistry	Hematology	Urinalysis
Sodium	Hematocrit	Appearance
Chloride	Hemoglobin	Volume
Potassium	MCH	Specific Gravity pH
CO2	MCHC	Glucose
BUN	MCV	Protein
Albumin	Total WBC	Microscopic evaluation of urinary sediment
Creatinine Clearance	Platelet Count	Trans-polydatin*
SGOT	Differential	Trans-resveratrol*
SGPT		
Total Bilirubin		
Direct Bilirubin		
Hep C		
HIV		
Trans-polydatin*		
Trans-resveratrol*		

*= Pharmacokinetic analysis only during timed blood/urine samples on trial days

Appendix 2 - Clinical Laboratory Normal Results

Parameter	Normal Range
Sodium	135-145 mmol/L
Chloride	98-108 mmol/L
Potassium	3.5-5.0 mmol/L
CO2	21-32 mmol/dL
BUN	10-20 mg/dL
Uric Acid	3.6-8.3 mg/dL
Albumin	3.5-5.0 g/dL
Creatinin	0.6-1.2 mg/L (Male) 0.5-1.1 mg/L (female)
SGOT	8-40 IU/L (male) 6-34 IU/L (Female)
ALT	10-50 IU/L (male) 5-38 IU/L (female)
Bilirubin	0.05-1.2 mg/dL
Serum total protein	6-8.5 g/dL
Glucose	4.4-6.1 mmol/L
Neutrophil	> 1500/mm3
Hematocrit	42-54% (Male) 38-46% (Female)
Hemoglobin	11.5-19.0 g/dL (Male) 10.5-17.5 g/dL (Female)
MCH	30-37 g/dL
MCHC	4.9-5.5mmol/L
MCV	80-99 fL
White blood cells.	0.4-1.1 x10^10/L
Platelets	120,000-450,000/mm3

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Vita

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Educational Institutions

Center College	2004-2008	Bachelor of Science	Biochem and Molecular Biology
University of Kentucky	2008-2012	PharmD	Doctor of Pharmacy
University of Kentucky	2012-2016	PhD Pharm Sci	Clinical and Experimental Therapeutics

Professional Publications

1. Ensor M, Williams J, Banfield A, Smith R, Lodder R. Effect of BSN272 on Hyperlipidemia and Atherosclerosis in LDLr-/- Mice. WebmedCentral PHARMACEUTICAL SCIENCES 2016;7(11):WMC005227.

2. Banfield A, Ensor M, Williams, J, Smith R, Lodder R. 4-Week Toxicity and Toxicokinetic Oral Gavage Study with Polydatin in Rats. WebmedCentral TOXICOLOGY 2016;7(11):WMC005231

3. Williams J, Ensor M, Gardner S, Smith R, Lodder R (2015) BSN723T Prevents Atherosclerosis and Weight Gain in ApoE Knockout Mice Fed a Western Diet. WebmedCentral ATHEROSCLEROSIS 2015;6(12):WMC005034.

4. Ensor M, Banfield AB, Smith RR, Williams J, Lodder RA (2015) Safety and Efficacy of D-Tagatose in Glycemic Control in Subjects with Type 2 Diabetes. *J Endocrinol Diabetes Obes* 3(1): 1065.

5. Ensor M, Williams J, Smith RR, Banfield AB, Lodder RA (2014) Effects of Three Low-Doses of D-Tagatose on Glycemic Control Over Six Months in Subjects with Mild Type 2 Diabetes Mellitus Under Control with Diet and Exercise. *J Endocrinol Diabetes Obes* 2(4): 1057.

6. Williams J, Spitnale M, Lodder R (2013) The Effect of D-Tagatose on Fructose Absorption in a Rat Model. *J Develop Drugs* 2: 111. doi:10.4172/2329-6631.1000111.

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Professional Positions

Pharmacist	2012 – Current	Markey Cancer Center