COVER PAGE FOR PROTOCOL AND STATISTICAL ANALYSIS PLAN

Official Study Title: The Effect of TLR4 Inhibition in Obese and Type 2 Diabetic Subjects (Eritoran2)

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THE EFFECT OF TLR4 INHIBITION IN OBESE AND TYPE 2 DIABETIC SUBJECTS (NIH_ERIT-002)

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GRANT TITLE: ROLE OF TLR4 ON INSULIN RESISTANCE IN HUMAN SUBJECTS (R01DK080157)

Study Product: *ERITORAN (eritoran for injection, 6.5 mg)*

Protocol Number: *NIH_ERIT-002*

IND Number: *123955-0007*

IRB Tracking Number: *20140498H*

NCT#: *NCT02267317*

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List of Abbreviations

ERK - extracellular-signal related kinase
FFA - free fatty acid(s)
IKK - IκB kinase
JNK - c-Jun N-terminal kinase
LPS - Lipopolysaccharide
MAPK - mitogen-activated protein kinases
NFκB – Nuclear Factor κ B
OGTT – Oral Glucose Tolerance Test
T2DM – Type 2 Diabetes Mellitus
TLR4 – Toll like receptor 4

Study Summary

Title	The Effect of TLR4 Inhibition in Obese and Type 2 Diabetic Subjects
Protocol Number	NIH_ERIT-002
Phase	Phase II
Methodology	This is a double-blind, placebo-controlled, cross-over study. Obese nondiabetic, obese type 2 diabetic, and lean normal glucose tolerant (control group) will receive the TLR4 inhibitor eritoran (E5564/eritoran for injection) or placebo (vehicle) I.V. for 72 h. Insulin sensitivity will be assessed with insulin clamps. Measurements of inflammation will be performed in blood, skeletal muscle and adipose tissue.
Study Duration	This study is funded by a 4 year NIH grant. Each subject will undergo 6 study visits over a period ranging between 4 and 6 months.
Study Center(s)	Single-center: Audie L. Murphy VA Medical Center in San Antonio, TX
Objective	To determine whether pharmacologic inhibition of TLR4 with eritoran (E5564) improves/ameliorates baseline (pre-existing) measurements of inflammation and insulin resistance in obese nondiabetic and type 2 diabetic subjects.
Number of Subjects	Three groups: (i) Obese nondiabetic; (ii) Obese type 2 diabetic; and (iii) lean normal glucose tolerant (NGT). 20 completers per group; (30 consented per group assuming ~30% screen failure or withdrawals)
Inclusion Criteria	1) One of these 3 groups: (i) obese (BMI = 30-37 kg/m2) normal glucose tolerant; (ii) obese (BMI = 30-37 kg/m2) T2DM subjects; or (iii) lean (BMI <26 kg/m2) normal glucose tolerant; 2) Both genders; 3) Age = 18-65 years; 4) All ethnic groups; 5) Premenopausal women , non-lactating, and with a negative pregnancy test. Postmenopausal women on stable dose of or not exposed to hormone replacement for ≥6 months; 6) Hematocrit ≥ 34%, serum creatinine ≤ 1.4 mg/dl, normal electrolytes, urinalysis, and coagulation tests; Liver function tests up to 2X; In T2DM subjects, hemoglobin A1c should be ≤ 8.5%; 7) Stable body weight (±1.5%) for ≥ 3 months; 8) One or less sessions of strenuous exercise/wk for last 6 months.
Exclusion Criteria	1) Presence of glucose intolerance based on ADA criteria (lean/obese NGT); diabetics with HgA1c ≥8.5% will be excluded. 2) Current treatment with drugs known to affect glucose and lipid homeostasis; The only allowed antidiabetic agents will be sulfonylureas and metformin. Subjects on a stable dose of statin (> 3 months) are eligible; 3) Non-steroidal anti-inflammatory drugs or systemic steroid use for more than a week within 3 months; 4) Current treatment with anticoagulants (warfarin). Aspirin (up to 325 mg) and clopidogrel will be permitted if they can be held for seven days prior to the biopsies, in accordance with the primary physician; History of coagulopathy will be excluded. 5) History of heart disease (New York Heart Classification greater than class II; more than non-specific ST-T wave changes on the ECG), peripheral vascular disease, pulmonary disease, smokers; 6) Poorly controlled blood pressure (systolic BP>160, diastolic BP>90 mmHg); 7) Active inflammatory, autoimmune, infectious, hepatic, gastrointestinal, malignant, and/or psychiatric disease.
Study Product, Dose, Route, Regimen	Eritoran for injection, 12 mg intravenously (I.V.) every 12 h. Total of 6 doses (72 mg)
Duration of administration	72 h
Statistical Methodology	Means between treatment groups will be compared using a repeated-measures General Linear Model (GML) analysis.

1. Introduction

This document is a protocol for a human research study. This study is to be conducted according to Good Clinical Practice guidelines as adopted by FDA, applicable government regulations, and Institutional research policies and procedures.

1.1. Background

Molecular biology of insulin resistance. Insulin resistance at the level of skeletal muscle is one of the earliest abnormalities in the pathogenesis of type 2 diabetes (T2DM). A wide array of abnormalities distinguishes muscle of obese and T2DM subjects from muscle of lean non-diabetic individuals. Defects include reductions in insulin-stimulated IRS-1 tyr phosphorylation, PI 3-kinase activation, Akt and AS160 phosphorylation, and GLUT4 translocation (1, 2). These molecular abnormalities are strongly correlated with reduced glucose disposal (1, 3). Therefore, interventions designed to improve muscle insulin resistance are likely to be effective in preventing and treating T2DM. The liver also is a key organ for maintenance of normal glucose homeostasis, and hepatic insulin resistance is an early event in the natural history of T2DM (4). Improving hepatic insulin sensitivity also is a key strategy for diabetes prevention and treatment.

TLR4 signaling. Toll-like receptors (TLRs) are cell-surface receptors that generate immune responses to pathogens by activating a cascade of inflammatory events (5). TLR4, one of the best characterized TLRs, is highly expressed in immune cells (monocytes, macrophages), and insulin target cells including myocytes, adipocytes and hepatocytes. Lipopolysaccharide (LPS or endotoxin), an outer membrane component of Gram (-) bacteria is a potent agonist of TLR4 (6). The soluble protein LPS-binding protein (LBP) binds LPS in plasma and regulates LPS-dependent responses. As discussed in more detail below, in addition to LPS, saturated free fatty acids (FFA) from a nutritional/metabolic source also can function as TLR4 agonists (7, 8).

The structure of LPS is shown below (Figure 1). The lipid A moiety within LPS is a 1-6-linked disaccharide of glucosamine, acylated with R-3-hydroxylaurate or myristate. The 3-hydroxyl groups of these saturated fatty acids are further 3-*O*-acylated by lauric acid, myristic acid, or palmitic acid (6); if lipid A is deacylated it loses its endotoxic properties and acts as an antagonist against lipid A (9). This implies that fatty acids acylated in lipid A are key for ligand recognition and TLR4 activation.

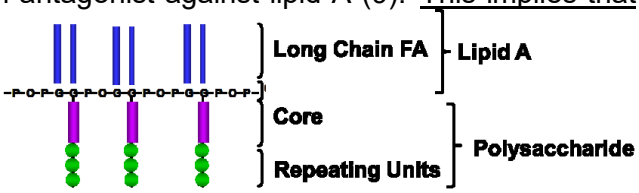


Figure 1. Chemical Structure of LPS

Upon stimulation of TLR4 and its coreceptors, CD14 and MD-2, the adaptor proteins TIRAP and MyD88 are recruited to the TIR domain of TLR4 (10). This association leads to the stimulation of inflammatory kinases, including the mitogen-activated protein kinases (MAPK); c-Jun N-terminal kinase (JNK), p38, and extracellular-signal related kinase (ERK), and the IκB kinase (IKK) complex, which then results in the activation of transcription factors such as nuclear factor-κB (NFκB), c-jun, and activator protein-1 (AP-1) (10). These inflammatory pathways have been linked to insulin resistance at a number of levels. Activation of JNK (11) and IKK (12) serine phosphorylates IRS-1, inhibiting its capacity to associate with PI-3 kinase. IKK also phosphorylates IκB, causing translocation of NFκB into the nucleus and gene transcription of inflammatory proteins such as tumor necrosis factor-α (TNFα), inducible nitric oxide synthase (iNOS), monocyte chemotactic protein (MCP)-1 and interleukin (IL)-6 (13). Concordantly, inhibition of either JNK or IKKβ/NFκB can improve insulin sensitivity (14-16). These findings provide evidence that the insulin signaling cascade is negatively regulated by TLR4.

Role of TLR4 on lipid-induced insulin resistance. Several lines of evidence implicate a deleterious effect of lipids *(i.e.* FFA) on insulin sensitivity. For example, most obese and T2DM subjects have elevated plasma FFA level that correlate with the severity of insulin resistance (17). The increase in plasma FFA is

caused by enhanced lipolysis, decreased lipid oxidation, and increased fat mass. In insulin resistant subjects, an acute reduction in plasma FFA with an antilipolytic drug (acipimox) significantly improves insulin sensitivity (18, 19), whereas an experimental elevation of plasma FFA in normal glucose-tolerant subjects rapidly induces insulin resistance (20, 21). Despite the considerable evidence implicating nutritional FFA in the pathogenesis of insulin resistance, how FFA impair insulin action, particularly in humans, is not well understood.

Based on the finding that saturated FA contained in lipid A moiety of LPS are essential for its biologic activity (9), Lee et al tested whether FFA from a nutritional/metabolic source have an LPS-like effect to directly activate TLR4. This group demonstrated that similar to lipid A, saturated FFA are potent activators of TLR4 in monocytes (7). In contrast, unsaturated FFA antagonize the effect of saturated FFA on TLR4 (22). In L6 myotubes, inhibition of TLR4 protects against the detrimental effect of FFA on insulin action (23, 24). During this funding cycle, our group showed that acute palmitate treatment induces robust NFκB activation via TLR4 in human myotubes (25). Studies in mice have shown that disrupted TLR4 function protects against acute and chronic fat-induced impairments in insulin action *in vivo* (26, 27). In general, studies have shown a protective effect of TLR4 inhibition against lipid-induced insulin resistance in muscle (26, 28). Some (26, 28), albeit not all (29) mouse studies that investigated the effect of TLR4 disruption also have revealed protection at the level of the liver.

The findings that saturated FFA can mimic the effect of LPS and activate TLR4 led us to evaluate during this funding period whether TLR4 expression and function are altered in insulin resistant subjects. We observed increased gene expression and protein content of TLR4 in muscle of obese and T2DM subjects, which inversely correlated with insulin sensitivity (25). We and others also demonstrated that TLR4 expression is elevated in mononuclear cells from insulin resistant subjects (30, 31). These data, along with the findings that insulin resistant subjects have increased plasma FFA concentration (17), and that FFA can stimulate TLR4 (7), suggest that TLR4 activation may be an important mechanism by which nutritional FFA impair insulin action.

Metabolic regulation by the microbiome. Initial research regarding the role of TLR4 in the pathogenesis of insulin resistance was conducted under the premise that saturated FFA from a nutritional/metabolic origin have an LPS-like effect to induce an inflammatory response. Notably, in the last ~3 years an increasing number of studies (some from our group) suggest that LPS produced by the gastrointestinal flora, *per se,* also could be a causative factor in human metabolic disease. The human gut hosts an enormous number and variety of microorganisms. This microbial community (microbiome) is considered to be a possible causative factor of metabolic disorders (32). In line with this, administration of non-absorbable antibiotics, which reduce gut flora number, improves systemic inflammation and glucose metabolism in *ob/ob* and high fat fed mice (33, 34). The mechanism by which gut microbiota regulates glucose metabolism is not clear. One hypothesis, discussed in more detail below, involves bacterial production of LPS (*i.e*. metabolic endotoxemia). Other potential mechanisms include the production of substances that alter intestinal wall integrity, food energy extraction, energy expenditure, and appetite (34-36),

Metabolic endotoxemia and insulin resistance. Accumulating evidence suggests that chronic elevation of circulating intestinal-generated LPS (*i.e.* metabolic endotoxemia) could play an important role in the pathogenesis of insulin resistance. We (37) and others (38, 39) have demonstrated that obese and T2DM subjects have elevated LPS concentration in plasma, in association with the severity of insulin resistance (37). Unlike other situations of endotoxemia, such as sepsis where plasma LPS level may increase ~100-fold (40), in situations of insulin resistance (obesity, T2DM) LPS levels are only modestly increased by ~2-3-fold. The cause of metabolic endotoxemia in obese and T2DM subjects is not clear. Ghanim et al. reported that a high calorie diet (high in fat and carbohydrate content) caused a significant elevation in plasma LPS, accompanied by increased TLR4 and NFκB expression in mononuclear cells (41, 42). In addition, a dietary survey conducted in 1,015 subjects demonstrated that high fat intake was directly associated with plasma LPS level (43). Postulated mechanisms by which fat intake may increase plasma LPS include (i) changes in the composition of the microbiome (33, 34, 44, 45); (ii) lipid-induced damage of the intestinal barrier (44), (iii) and chylomicron-mediated transport of LPS (46).

The observation that plasma LPS concentration is elevated during states of insulin resistance has prompted studies into the effect that LPS level modulation has on insulin sensitivity. As mentioned above, reducing LPS levels with non-absorbable antibiotics improves glucose metabolism in insulin resistant mice (33, 34). In line with this finding, intravenous low-dose LPS administration to healthy individuals rapidly induces insulin resistance (47). Collectively, these data suggest that metabolic endotoxemia could play an important role in the pro-inflammatory state and insulin resistance that occurs in obese and T2DM subjects.

1.2. Innovation

Insulin resistant subjects have increased TLR4 expression and signaling in insulin sensitive tissues (muscle) (25) and peripheral mononuclear cells (30, 31). Moreover, the plasma concentration of the TLR4 ligands, FFA (17) and LPS (37, 48, 49), is increased in these subjects. This suggests that activation of TLR4 by FFA and LPS may be a key mechanism underlying the inflammatory state and insulin resistance of obesity and T2DM. **Yet, there is no direct *in vivo* data demonstrating a role for TLR4 in the pathogenesis of insulin resistance in human subjects.** In this study we will examine the effect of a **selective TLR4 antagonist**, with the **goals** to (i) establish the role that TLR4 signaling plays in the pathogenesis of insulin resistance and T2DM in humans; and (ii) elucidate the mechanism by which TLR4 mediates insulin resistance in human subjects. **This is the first human study to determine the cellular and molecular effects of a TLR4 antagonist in insulin sensitive tissues (muscle, fat) *in vivo*, and to examine how TLR4 inhibition affects whole body glucose metabolism.** Figure 2 below outlines the proposed model by which TLR4 mediates insulin resistance and summarizes the rationale behind this application.

Figure 2. Working Model of TLR4- mediated insulin resistance. In insulin-resistant states (obesity, T2DM) there is an increase in the plasma concentration of two TLR4 ligands, LPS and FFA. LPS is elevated due to increased intestinal permeability and chylomicron-mediated transport. FFA are elevated due to lipolysis and adipose tissue expansion.

UponTLR4 stimulation, the IKK/NFkB and MAPK pathways are stimulated. These inhibit insulin signaling at the level of IRS-1 and promote gene transcription of pro-inflammatory proteins that also impair insulin action.

This protocol (NIH_ERIT-002) will test whether a **selective TLR4 antagonist** will improve/ameliorate baseline (pre-existing) inflammation insulin resistance.

1.3. Preliminary data. Below is a description of preliminary data which lay the foundation for the proposed research.

a) Insulin resistant subjects have abnormal TLR4 expression and TLR4-driven signaling in skeletal muscle. Considering that animal models of insulin resistance are characterized by increased TLR4 expression (26), we examined whether insulin resistant subjects also have alterations in the expression and function of this receptor. We demonstrated that obese and T2DM subjects have increased TLR4 gene expression (Fig. 3A), TLR4 protein content (Fig. 3B) and inflammatory gene (IL-6) expression (Fig. 3C) in muscle, indicative of increased TLR4-driven signaling (25).

Fig. 3A

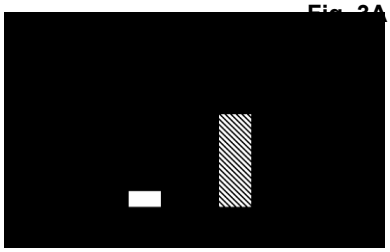


Fig. 3B

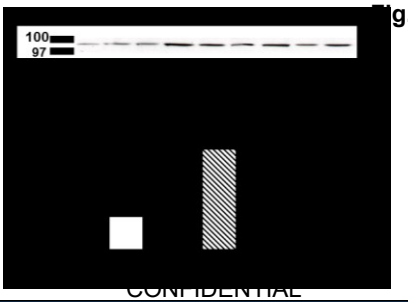


Fig. 3C

b) An experimental elevation in circulating FFA increases TLR4 expression/signaling in muscle. We showed that saturated FFA increase TLR4 expression and signaling in primary human myotubes (25). To determine whether lipids also promote an inflammatory response in humans *in vivo*, we administered a continuous (48 h), low dose lipid infusion (Intralipid 30 ml/h) to 14 lean normal glucose-tolerant subjects. As a control experiment, on a different occasion the same subjects received saline. The lipid infusion raised mean plasma FFA concentration from 355 to 484 μM [a typical plasma FFA level observed in insulin resistant subjects (450-600 μM)] and reduced peripheral insulin sensitivity (assessed by hyperinsulinemic euglycemic clamp) by 17%. Molecular analysis of *vastus lateralis* muscle demonstrated that raising FFA upregulated the expression of TLR4 by ~50% (50). Lipid infusion also increased the expression of various other genes within the TLR pathway (Fig. 4A) and impaired insulin-mediated IRS-1 tyr phosphorylation (Fig. 4B) (50). Notably, mass spectrometry-based analysis revealed that the total (unfractionated) muscle content of several intracellular lipid species (ceramides, diacylglycerols, acylcarnitines) was unaffected by Intralipid (50). <u>These data demonstrate that a modest increase in plasma FFA is sufficient to upregulate the TLR4 pathway (and induce insulin resistance) in healthy subjects.</u>

Fig. 4A

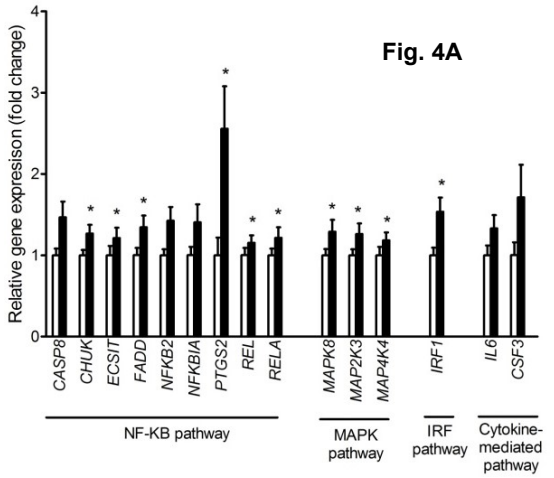
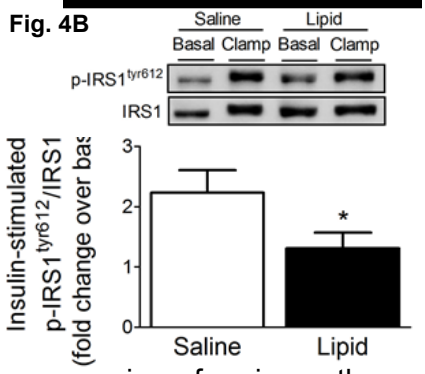


Figure 4. A lipid infusion (black) increases TLR4-related genes (A) and inhibits IRS-1 try phosphorylation (B). *P<0.05 vs Saline (white)

Fig. 4B



c) TLR4 mediates FFA-induced insulin resistance in myotubes. To evaluate whether the inflammatory response caused by FFA is mediated via TLR4, we tested the effect of the TLR4 inhibitor TAK-242 (Takeda Pharm.) on FFA-induced NFκB p65 and JNK phosphorylation, and IL-6 expression in L6 muscle cells (23). Stearate (400 μM for 6 h), a saturated FFA, increased NFκB p65 phosphorylation (Fig. 5A), JNK phosphorylation (Fig. 5B), and IL-6 mRNA level (Fig. 5C). These inflammatory responses were significantly reduced by TAK-242, although the inhibition of NFκB and JNK phosphorylation was partial.

Fig. 5A

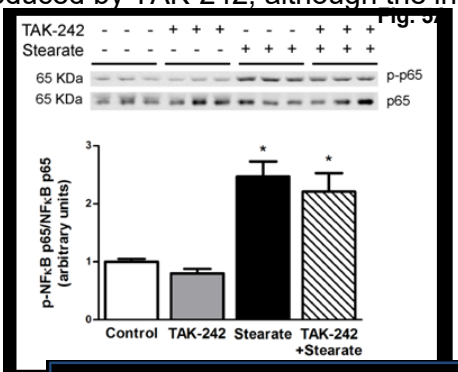


Fig. 5B

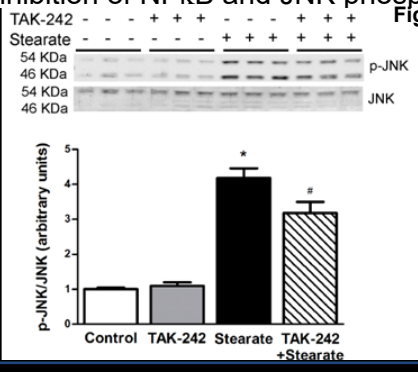
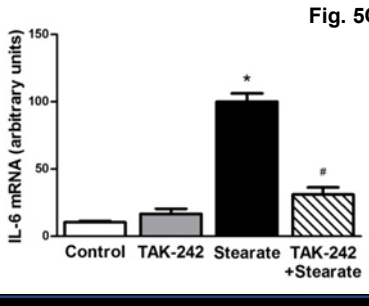
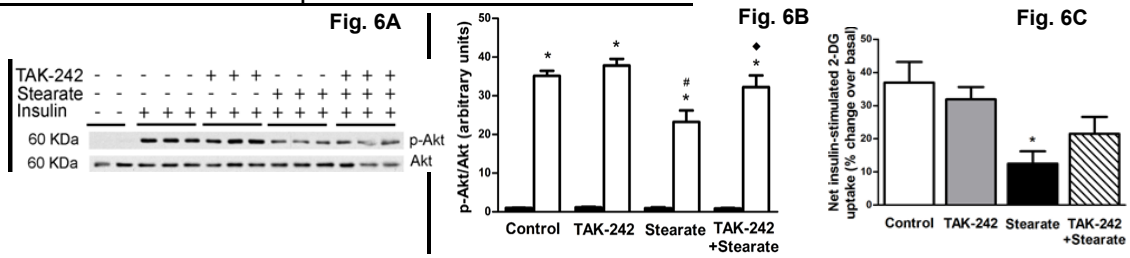


Fig. 5C



We also tested the effect of TAK-242 on the insulin transduction pathway and glucose transport in L6 myocytes (insulin was dosed 10 nM for 5 min and 100 nM for 20 min, respectively) (23). Stearate (400 μM for 6 h) reduced insulin-stimulated Akt phosphorylation (Figs. 6A and 6B). Notably, the reduction in Akt phosphorylation was no longer observed when cells were pre-treated with TAK-242. Stearate (400 μM for 6 h) also reduced insulin-stimulated glucose transport (Fig. 6C). TAK-242 restored glucose transport, such that the reduction by stearate was no longer statistically significant. These *in vitro* studies demonstrate that pharmacologic TLR4 inhibition ameliorates lipid-induced insulin resistance.

Figure 6. Effect of TAK242 on FFA-induced insulin resistance in myotubes. 6A shows Western blots and 6B shows graphical data on Akt phosphorylation; *P<0.05 vs. basal within group, #P<0.05 vs. control, ♦P<0.05 vs. stearate. 6C shows glucose transport data; *P<0.05 vs. control.



d) Elevated TLR4 content in peripheral blood mononuclear cells (PBMC) from insulin resistant subjects. Immune cells can infiltrate insulin sensitive tissues (muscle, liver, fat) where they induce an inflammatory response that impairs insulin action. We examined whether TLR4 content/signaling is altered in PBMC from insulin resistant subjects. As shown in Figure 7, reproduced from (31), PBMC from obese and T2DM subjects have increased TLR4 expression (Fig. 7A) and signaling (ERK phosphorylation) (Fig. 7B).

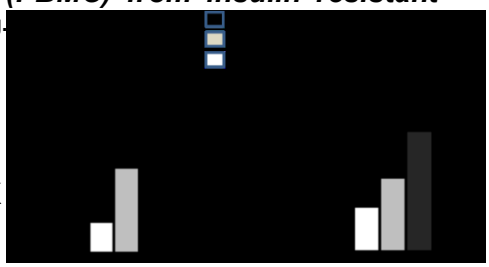


Figure 7. TLR4 expression (A) and ERK phosphorylation (B) in PBMC. n=8-17group. *P<0.05 vs. lean

e) Lipid infusion increases TLR4 expression/signaling and TLR4-regulated cytokines. We have demonstrated that exposure to lipids increases TLR4 expression/signaling in human muscle, both *in vitro* (25) and *in vivo* (50). Next, we examined whether the increases in TLR4 expression and signaling observed in immune cells from insulin resistant subjects also are caused by elevated FFA. Using flow cytometry we measured TLR4 cell surface expression and MAPK phosphorylation in blood monocytes from the lean normal-glucose tolerant subjects described above (Fig. 4) who received Intralipid/saline for 48 h. Infusion of lipid (but not saline) significantly increased TLR4 expression (Fig. 8A) and the phosphorylation of JNK (Fig. 8B) and p38 (Fig. 8C). Notably, Intralipid also enhanced LPS-induced JNK phosphorylation when blood monocytes were exposed to LPS (1 μg/ml) *ex vivo* (Fig. 8D), suggesting that lipids "prime" the monocytes to endotoxin.

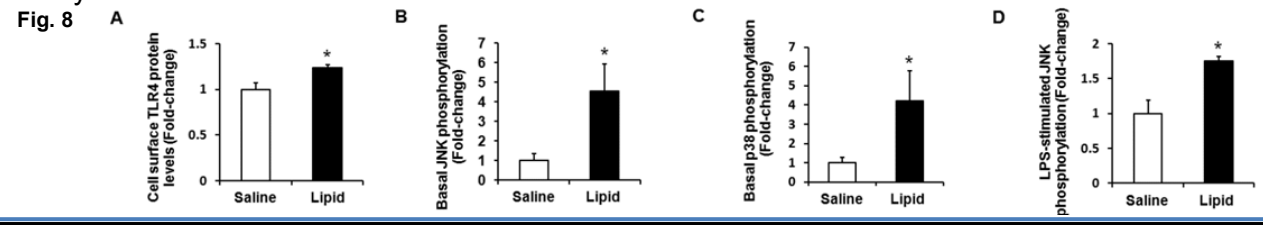


Figure 8. Effect of saline/lipid infusion on TLR4 cell surface expression and MAPK phosphorylation in monocytes. A: TLR4 cell surface expression. B: Basal JNK phosphorylation. C: Basal p38 phosphorylation. D: LPS-stimulated JNK phosphorylation. *P<0.05 vs. Saline

f) Lipid infusion increases plasma cytokine concentration. As shown in Figure 9, the plasma TNFα concentration was significantly elevated after the infusion of Intralipid, but not saline. Interestingly, the plasma level of other cytokines such as IL-6 and MCP-1 was not different after lipid infusion, suggesting a selective (*i.e.* regulated) effect of the lipid infusion on cytokine production/release.

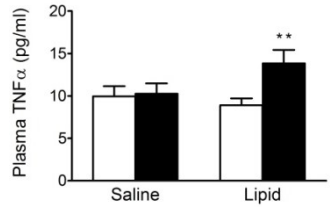


Figure 9. A prolonged lipid infusion increases plasma TNFα level (n=12/group). ☐ Before ■ After. **P<0.05 vs. post-saline and vs. pre-lipid

g) Plasma LPS concentration is increased in obese and T2DM individuals. We measured LPS concentration in plasma from 11 lean, 9 obese nondiabetic, and 10 obese T2DM subjects. Insulin sensitivity (M) was measured with a euglycemic clamp. As shown in Fig. 10A, plasma LPS concentration was significantly elevated in the obese and T2DM subjects. Furthermore, there was a negative correlation between LPS concentration and insulin sensitivity (10).

Fig. 10A

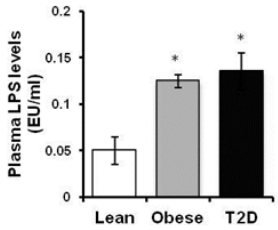


Fig. 10B

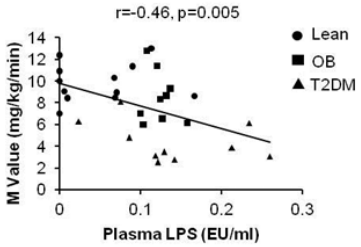


Figure 10. Plasma LPS concentration in humans (A) and correlation with insulin sensitivity (M value) (B). *P<0.05 vs. lean

h) LPS impairs insulin action *ex vivo*. We utilized an isolated muscle preparation to evaluate the effect of LPS on insulin signaling and glucose (2DG) transport *ex vivo*. Rat *epitrochlearis* muscles were incubated for 4 h with LPS (100 ng/ml or 1 μg/ml), followed by 50 mU/ml insulin (20 min). A shown in Figure 11, LPS reduced insulin signaling (Fig. 11A) and glucose transport (Fig. 11B) in a dose-dependent manner.

Fig. 11A

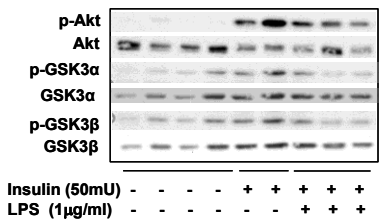


Fig. 11B

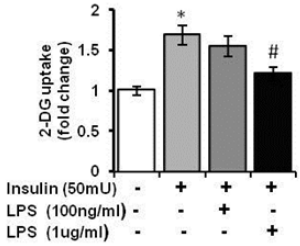
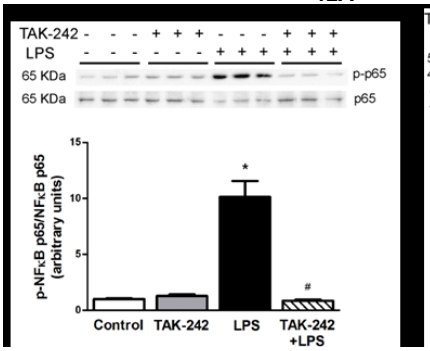


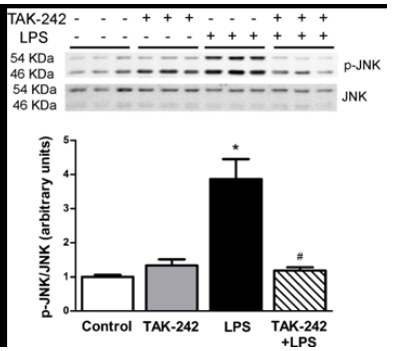
Figure 11. Effect of LPS on insulin signaling (A) and glucose uptake (B) in rat *epitrochlearis* muscle *ex vivo* (n=4-6)/group. *P<0.05 vs. basal, #P<0.05 vs. insulin

We tested the effect of the TLR4 inhibitor TAK-242 on LPS-induced NFκB p65 and JNK phosphorylation in L6 muscle cells (Fig. 12). LPS (100 ng/ml for 1 h) increased NFκB (Fig. 12A) and JNK phosphorylation (Fig. 12B) and these responses were completely inhibited by TAK-242 (1 μM) (23). In line with these findings, TAK-242 prevented LPS-mediated impairments in insulin-stimulated Akt phosphorylation (Fig. 12C) and glucose transport (Fig. 12D) (23).

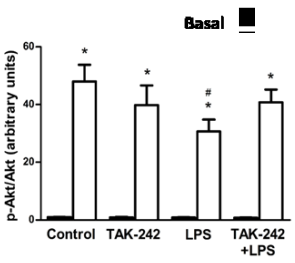
12A



12B



12C



12D

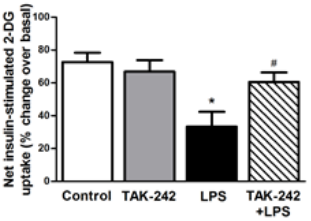


Figure 12. Pharmacologic blockade of TLR4 reduces LPS-induced NFκB (A) and JNK (B) phosphorylation, and protects against LPS-induced insulin resistance (C and D). In A, B, and D *P<0.05 vs. control, #P<0.05 vs. LPS; In C *P<0.05 vs. basal (no insulin), #P<0.05 vs. control; Adapted from *Hussey et al, Biosci Rep. 2012 ;33(1):37-47.*

i) Pharmacologic inhibition of TLR4 improves peripheral insulin resistance in vivo. To examine whether pharmacologic inhibition of TLR4 improves glucose metabolism *in vivo*, we tested the effect of the competitive TLR4 antagonist eritoran (E5564) (kindly provided by Eisai Pharmaceuticals, Andover, MA) on lipid-induced insulin resistance using a 2-step euglycemic clamp with tritiated glucose, as described (51).

Male Sprague-Dawley rats (~200 g) were pre-treated with eritoran (5 mg/kg) or vehicle through an arterial catheter.15 min after eritoran administration, Intralipid 20% (8.5 mg/kg.min) or saline were infused for 8 h. ~4 h after the first dose, a second dose of eritoran (5 mg/kg) or vehicle was given. The clamp was started 15 min after the second dose (time +4h) of eritoran/vehicle. During Step 1 (0-120 min), insulin was infused at 0.4 mU/kg.min to measure hepatic insulin sensitivity [suppression of endogenous glucose production (EGP)]. During Step 2 (120-240 min), insulin was infused at 4 mU/kg.min to measure peripheral (muscle) insulin sensitivity. A variable dextrose infusion was administered to maintain glucose at 5 mM. Somatostatin (3 μg/kg.min) was infused during the clamp to suppress endogenous insulin release. The protocol is shown in Figure 13.

Fig. 13

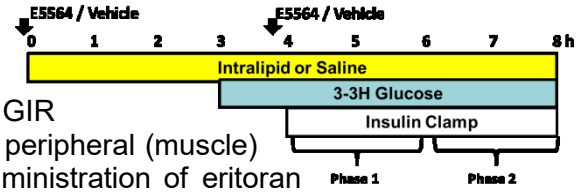


Figure 14A below shows the glucose infusion rate (GIR) required to maintained euglycemia during the clamp. The mean GIR during the last 30 min of Phase 2 (210-240 min), an indication of peripheral (muscle) insulin sensitivity, was reduced by the lipid infusion, and the administration of eritoran significantly ameliorated the reduction in GIR caused by the lipid (Fig. 14A). Consistent with these findings, eritoran reversed lipid-induced reductions in peripheral (muscle) glucose disposal during Phase 2 of the clamp (Fig. 14B).

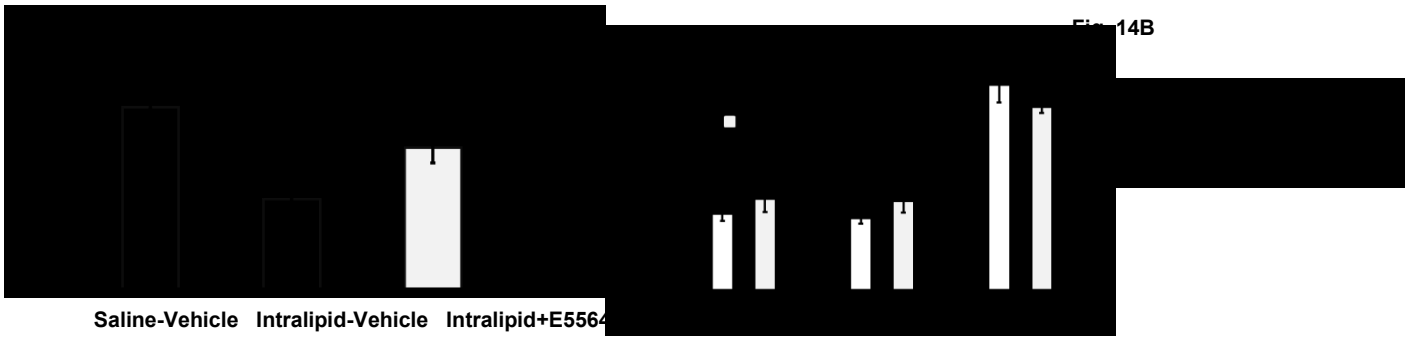


Figure 14. Effect of the TLR4 inhibitor E5564 on glucose infusion rate (A) and insulin-mediated glucose disposal (B) in rats. n=4-6 /group.

j) Eritoran ameliorates lipid-induced insulin resistance in the liver. Figure 15 shows the effect of eritoran (E5564) on lipid-induced hepatic insulin resistance. As expected, in saline-infused rats insulin caused a dose-dependent suppression of EGP (from Step 1 to Step 2). However, insulin was unable to inhibit EGP in lipid-treated rats. Notably, eritoran restored the ability of insulin to suppress EGP

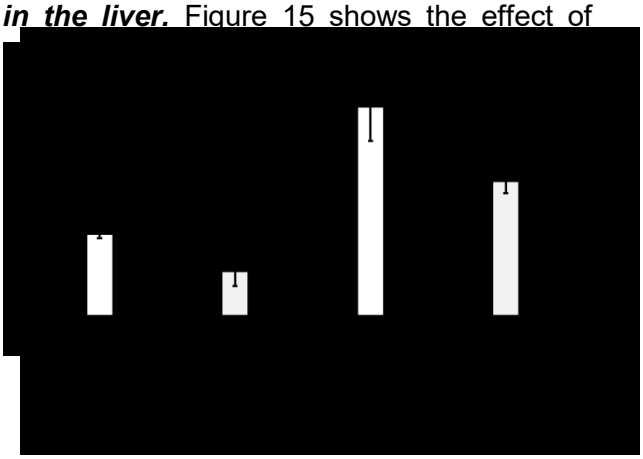


Figure 15. Effect of the TLR4 inhibitor E5564 on hepatic insulin sensitivity. Data are percent reduction (from baseline) in HGP caused by insulin. n=4-6 /group.

In summary, our preliminary data demonstrate that pharmacologic inhibition of TLR4 protects against lipid- and LPS-induced insulin resistance *in vitro*. Moreover, **pharmacologic blockade of TLR4 with eritoran in rats partially reverses the deleterious effect of a lipid infusion in both the periphery (muscle) and liver.** Based upon these exciting findings obtained from pre-clinical studies, a proof-of-concept experiment is proposed to test the *hypothesis* that pharmacologic inhibition of TLR4 will reduce inflammation and improve baseline (*i.e.* pre-existing) impairments in glucose metabolism in insulin resistant (obese and T2DM) subjects.

1.4. Investigational Agent

Eritoran (E5564) is a drug originally developed by Eisai Pharmaceuticals (eritoran tetrasodium, currently labeled “eritoran for injection”) for the treatment of sepsis. Eritoran is a synthetic analogue of Lipid A, the toxic part of endotoxin (also known as lipopolysaccharide, LPS). Eritoran has been shown to block the effects of LPS *in vitro, in vivo,* and in humans. The mechanism of action of eritoran is competitive inhibition of the binding of LPS to its functional receptor complex of TLR4 and co receptor MD2 (52-55). The pharmacokinetic and pharmacodynamic properties and safety profile of eritoran have been studied extensively during phase 1, 2 and 3 experimental studies of endotoxemia in healthy volunteers, patients with severe sepsis, and patients with impaired hepatic function (55-58). Eritoran is safe and well tolerated, even at the highest doses tested (55). We have confirmed the ability of eritoran to improve glucose metabolism in lipid-treated rats (Preliminary Data; Figures 14-15) and to suppress LPS- and stearate-mediated MAPK activation in primary human myotubes (Figure 16).

Figure 16. Effect of the TLR4 Inhibitor E5564 on p38 MAPK phosporylation In human myotubes. Primary human myotubes derived from a lean healthy subject were pre-treated with 100 nm E5564 for 15 min, followed by stimulation with 100 ng/ml LPS for 1h (A) or 400 μM stearate for 6 h (B).

1.5. Dose Rationale and Risk/Benefits

Eritoran (E5564) has a half-life of ~25-30 h and pharmacodynamic studies have shown that ~12 h after eritoran administration (12 mg), the inhibitory effect of the drug on TLR4 signaling is fully maintained (59). This dose (12 mg) is safe and well tolerated, and higher doses do not confer further inhibitory effect on TLR4 (59).

This study is not intended to directly benefit the subject participants. The proposed research is expected to expand our knowledge on the pathophysiology of insulin resistance and T2DM. This enhanced knowledge may lead to the development of novel treatments for these disorders.

The pharmacokinetic and pharmacodynamic properties and safety profile of eritoran have been studied extensively during phase 1, 2 and 3 experimental studies of endotoxemia in healthy volunteers, patients with severe sepsis, and patients with impaired hepatic function [57-60]. Eritoran is safe and well tolerated, even at the highest doses tested [57]. The only adverse event that occurred at a significantly higher rate with eritoran was phlebitis, although phlebitis occurred mainly at the highest doses tested (higher than what is proposed in this study) and during continuous infusion. Phlebitis was minimal with intermittent administration (as given in this study) and was transient. Risks will be minimized by a careful screening process including medical history, physical exam, and review of complete blood count, coagulation tests, chemistry and electrocardiogram. The presence of experienced personnel, and of at least one physician, at all times is also critical to reducing risks.

2. Study Objective

To determine whether pharmacologic inhibition of TLR4 with eritoran will reduce inflammation and improve glucose metabolism in insulin resistant (obese and T2DM) subjects. *Rationale.* Insulin resistant subjects have increased TLR4 expression and signaling (MAPK, NFκB) in insulin sensitive tissues (25) and inflammatory cells (25, 30, 31). Moreover, the plasma concentration of two endogenous TLR4 ligands, FFA (17) and LPS (37, 48, 49), is increased in these

individuals. This suggests that activation of TLR4 by FFA and LPS may be a mechanism underlying the inflammatory state and insulin resistance of obesity and T2DM. Yet, the relevance of TLR4 to the etiology of human metabolic disease is not known. In this Aim, we will test the hypothesis that TLR4 plays a direct role in the pathogenesis of insulin resistance in humans. Thus, we will examine whether blocking TLR4 with eritoran ameliorates the inflammatory state and improves glucose metabolism in insulin resistant (obese and T2DM) subjects. **This is the first human study to determine the molecular effects of a TLR4 antagonist in insulin sensitive tissues (muscle, fat) *in vivo*, and to examine how TLR4 inhibition affects whole body glucose metabolism.**

3. Study Design

3.1. Subjects

20 obese (BMI = 30-37 kg/m2) normal glucose-tolerant, 20 obese (BMI = 30-37 kg/m2) T2DM subjects, and 20 lean (BMI <26 kg/m2) normal glucose-tolerant (control) subjects will be enrolled (~50% males). Subjects will be recruited through local advertisement and through the Diabetes and General Medicine Clinics of the Audie L. Murphy Division of the STVHCS and the Texas Diabetes Institute in San Antonio, TX. ~90 subjects will be consented assuming ~30% screen failure or withdrawals.

3.2. General Design

The study is designed as a Phase II, randomized, controlled, interventional study. Enrollment duration is planned over a 4-year period. Each subject will undergo 6 study visits over a period of 4-6 months.

Study Design.

Following a screening period, which can be up to three months, each subject will be admitted on 2 different occasions (separated by 4-10 weeks each) to receive either eritoran or vehicle (in random order) for 72 h. Studies will be performed in the Bartter Research Unit (BRU), located in the Audie L. Murphy VA Medical Center in San Antonio, TX.

3.3. Study Endpoints

Primary outcome: Effect of eritoran on muscle insulin action (insulin signaling and peripheral glucose disposal) and hepatic insulin sensitivity (insulin suppression of EGP) in obese and T2DM subjects.

Secondary outcomes: Effect of eritoran on:
- *Inflammatory signaling in muscle and adipose tissue*: TLR4 signaling and inflammatory gene expression
- *Muscle and adipose tissue macrophage infiltration*.
- *TLR4 expression, NFκB and MAPK activation/phosphorylation on peripheral blood monocytes*.
- *Plasma cytokine concentration*.
- *Intramyocellular diacylglycerol and ceramide content (membrane and cytoplasm fractions).*

3.4. Potential Risks to Subject Safety

a) Drug Administration.
Insulin and Glucose: Since the glucose infusion is designed to counterbalance the metabolic effects of insulin, the risk of hypoglycemia is very low. No other side effects of insulin and glucose are known. Plasma glucose concentration will be determined at 5 min intervals throughout the period of insulin/glucose administration.

Eritoran: Six (6) phase I studies evaluating the safety of eritoran in healthy subjects have been conducted (55-60). The only adverse event that occurred at a significantly higher rate with eritoran was phlebitis, although phlebitis occurred mainly at the highest doses tested (higher than what is proposed in this study) and during continuous infusion. Phlebitis was minimal with intermittent administration (as given in this study) and was transient.

The pharmacokinetic and pharmacodynamic properties and safety profile of eritoran have been studied extensively during phase 1, 2 and 3 experimental studies of endotoxemia in healthy volunteers, patients with severe sepsis, and patients with impaired hepatic function (55-58). Eritoran is safe and well tolerated, even at the highest doses tested (55).

b) Blood withdrawal. All studies involve the withdrawal of blood. Any subject who has donated blood in the previous two months will not be studied. The subjects will be instructed not to donate blood for two months after the study. Any subject with a hematocrit of less than 34% will not be studied. The maximal amount of blood to be drawn during the entire study for any given subject will be approximately $\leq$ 534 mL. Hematology comprehensive metabolic panel with lipids and liver function tests will be obtained at the Infusion Visit to assess effects on blood cell count, liver function and lipid metabolism.

c) IV lines. Intravenous catheters will be used for infusions and venipuncture. Local hematomas occur in about 1% of catheterizations. Infection is possible (<1%), but we have not experienced this complication. One instance of thrombophlebitis has been observed (<1%). The hand with the catheter will be placed in a warm (55^{o} C) transparent plastic box to arterialize venous blood. We have observed one instance of skin burning (2nd degree) using these boxes (<0.01%).

d) Muscle biopsy. At the time of biopsy, subjects may feel pain, discomfort, or pressure (variably described by different subjects) for about 5-10 seconds. Pain or discomfort ceases as soon as the cannula is withdrawn. Local hematomas occur in <2% of subjects. One patient experienced a moderately painful hematoma that resolved within 2 weeks (0.1%). About 1 in 50 subjects report non-clinically evident numbness or altered sensation at the biopsy site, which is transient.

There is the possibility that a future biopsy may not be done at the discretion of the PI in case the subject did not tolerate well a prior biopsy.

e) Adipose tissue biopsy. A burning discomfort will be felt for about 5-10 seconds during the application of lidocaine. Mild wound infections occur in <2% subjects.

f) Subjects will be exposed to radioactivity during these studies. The radiation exposure from tritiated glucose and the DEXA is well within guidelines (Shreve WW et al. Proc 2nd International Conference on Peaceful Uses of Atomic Energy, Geneva, 1958; U.S. Department of Commerce National Bureau of Standards Handbook 69, 1969). Subjects will also be exposed to a very small amount of radiation during the DEXA exam (0.04 mrem), which equals one day of natural background radiation. The cumulative radiation exposure is well within the dose range (1,000 μCi) approved by the Hospital Radiation Committee. The local VA Radiation Committee will approve these studies before anyone is enrolled. At these low exposures, risk is minimal.

4. Subject Selection and Withdrawal

4.1. Inclusion Criteria

1) Subjects capable of giving informed consent.
2) One of these 3 groups: (i) obese (BMI = 30-37 kg/m2) normal glucose tolerant; (ii) obese (BMI = 30-37 kg/m2) T2DM; or (iii) lean (BMI <26 kg/m2) normal glucose tolerant. Lean subjects have no family history of diabetes.
3) Both genders.
4) Age=18-65 years. Older subjects are excluded because aging is a pro-inflammatory state.
5) All ethnic groups.
6) Premenopausal women, non-lactating, and with a negative pregnancy test. Postmenopausal women on stable dose of or not exposed to hormone replacement for ≥6 months.
7) Lab: Hematocrit ≥34%, serum creatinine ≤1.4 mg/dl, normal electrolytes, urinalysis, and coagulation tests. Liver function tests up to 2X normal range.
8) Stable body weight (±1.5%) for ≥3 months.
9) One or less sessions of strenuous exercise/wk for last 6 months.

4.2. Exclusion Criteria

1) For non-diabetic subjects, the presence of glucose intolerance on OGTT based on American Diabetes Association (ADA) criteria will be exclusionary
2) Current treatment with drugs known to affect glucose and lipid homeostasis. Subjects on a stable dose of statin (>3 months) are eligible. Subjects on sulfonylureas and/or metformin are eligible.
3) Hemoglobin A1c ≥ 8.5% (diabetic subjects only).
4) Non-steroidal anti-inflammatory drugs or systemic steroid use for more than 1 week within 3 months
5) Current treatment with anticoagulants (warfarin). Aspirin (up to 325 mg) and clopidogrel will be permitted if these can be held for seven days prior to the biopsies. History of coagulopathy will be excluded.
6) History of heart disease (New York Heart Classification greater than class II; more than non-

specific ST-T wave changes on the ECG), peripheral vascular disease, pulmonary disease, smokers.

7) Poorly controlled blood pressure (systolic BP>160, diastolic BP>90 mmHg).
8) Active inflammatory, autoimmune, infectious, hepatic, gastrointestinal, malignant, and psychiatric disease.

4.3. Subject Recruitment and Screening

Subjects will be recruited by advertisement and through the outpatient clinics of the South Texas Veterans Health Care System and the Texas Diabetes Institute (TDI) in San Antonio, TX. Every month, ~25 newly diagnosed T2DM patients are referred to the TDI.

Screening will be performed during Visits 1 and 2. On Visit 1, following an overnight fast (no food after 9 PM) subjects will come to the BRU at approximately 7 AM for consenting, followed by screening procedures including a medical history, physical exam, and screening tests (CBC, chemistry, lipids, HbA1c, PT, PTT, urinalysis and ECG). For subjects who are eligible based on these screening tests, an oral glucose tolerance test (OGTT) will be performed on Visit 2.

4.4. Early Withdrawal of Subjects

4.4.1. When and How to Withdraw Subjects

Subjects have the right to withdraw fully or partially from the study at any time and for any reason without prejudice to his or her future medical care by the physician or at the institution.

Withdrawal of full consent for a study means that the subject does not wish to receive further investigational treatment and does not wish to or is unable to continue further study participation including any follow-up in person, by phone, through third parties including relatives or friends, via discussion with other treating physicians, and by use of medical records; subject data up to withdrawal of full consent will be included in the analysis of the study. Any subject may withdraw full consent to participate in the study at any time during the study. The Sponsor Investigator or sub-investigator will discuss with the subject appropriate procedures for withdrawal from the study.

4.4.2. Data Collection and Follow-up for Withdrawn Subjects

If subjects are withdrawn prematurely from the study, appropriately designated research staff will make efforts to collect at least survival data throughout the protocol defined follow-up period for that subject.

Sponsor Investigator will consult with Study Statistician with regard to any incomplete data set as compared to the full data set that fully supports the analysis. If a subject withdraws consent to participate in the study, attempts will be made to obtain permission to record survival data up to the protocol-described end of subject follow-up period.

Sponsor Investigator and designated research staff make it a high priority to obtain survival data on all subjects lost to follow up. Lost to follow up will be defined as a subject missing 2 or more consecutive visits, not answering or responding to 3 follow up phone calls to subject or emergency contacts, or returned receipt of 1 certified letter.

5. Study Drug

5.1. Description

Eritoran (eritoran for injection) is a drug originally developed by Eisai Pharmaceuticals for the treatment

of sepsis. Eritoran is a synthetic analogue of Lipid A, the toxic part of endotoxin (also known as lipopolysaccharide, LPS). Eritoran has been shown to block the effects of LPS *in vitro, in vivo,* and in humans. The mechanism of action of eritoran is competitive inhibition of the binding of LPS to its functional receptor complex of TLR4 and co receptor MD2 (52-55). The pharmacokinetic and pharmacodynamic properties and safety profile of eritoran have been studied extensively during phase 1, 2 and 3 experimental studies of endotoxemia in healthy volunteers, patients with severe sepsis, and patients with impaired hepatic function (55-58). Eritoran is safe and well tolerated, even at the highest doses tested (55). All studies on eritoran have been conducted using the intravenous route because this drug was developed for treatment of sepsis in critically-ill patients.

Eritoran tetrasodium is a sterile, white freeze-dried cake or powder containing 7 mg of eritoran tetrasodium (as the tetrasodium salt) or 6.5 mg of eritoran (as the free acid) active packaged in a 10-mL glass vial. Since these will be a double-blind studies, matching placebo vials will be used.

5.2. Treatment Regimen

Following a screening period, which can be up to one month, each subject will be admitted on 2 different occasions (separated by 3-6 weeks each) to receive either eritoran or vehicle (in random order) for 72 h. Eritoran will be dosed 12 mg I.V. every 12 for 72 h. A total of 6 doses (72 mg total) will be administered. Each dose is diluted in 100 ml of 5% Dextrose (D5W) and infused over 4 hours.

Studies will be performed in the Bartter Research Unit (BRU), located in the Audie L. Murphy VA Medical Center in San Antonio, TX.

5.3. Method for Assigning Subjects to Treatment Groups

The randomization key will be designed by the VA Research Pharmacy at the Bartter Clinical Research Unit (BRU) and provided in a blinded fashion to the Clinical Study Coordinator. The Study Coordinator stores the Enrollment key, which is kept in a locked cabinet. Study staff maintains specimens in a de-identified manner using only the unique Subject ID/Randomization number; source documents and data records are stored in electronic systems with restricted access and paper records in locked cabinets.

5.4. Preparation and Administration of Study Drug

The study drug will be provided by Eisai under Material Transfer Agreement (Attachment A., Section 15) and shipped directly to the Audie L. Murphy Hospital Research Pharmacy. Upon receipt of physician's order, the IV infusion at required dose will be prepared by the Research Pharmacist, according to VA policies and procedures and dispensed to designated study staff who will administer the infusion. See also Blinding Study Drug, Section 5.8 and Study Procedures, Section 6.3, Visit 3 Infusion Protocol for further details.

5.5. Subject Compliance Monitoring

Subject compliance is demonstrated by adherence to instructions in the consent and appearing for all study visits and procedures. Subjects receive a copy of the informed consent form at the time of consenting and will have an opportunity to review the consent document at every visit.

Study staff will call via telephone 1-2 days before each visit and provide them with instructions (*e.g*. arrive fasting, bring concomitant medications, *etc*).

5.6. Prior and Concomitant Therapy

Exclusion criteria:

Drugs that affect glucose and lipid homeostasis (subjects on a stable dose of statin >3 months are

eligible; subjects on sulfonylureas and/or metformin are eligible).

Non-steroidal anti-inflammatory drugs or systemic steroid use for >1 week within 3 months;

Treatment with anticoagulants (warfarin). Aspirin (up to 325 mg) and clopidogrel will be permitted if these can be held for seven days prior to the biopsies.

5.7. Packaging

Per MTA, Eisai will supply a total of 1,220 7mg vials of Eritoran for injection for use in the study. The Audie L. Murphy VA Hospital Research Pharmacy ("VA Research Pharmacy") will keep all Study Drug in a locked, secure area at all times.

Nature and Contents of the Container
Eritoran for injection is packaged as unpreserved sterile lyophilized drug product in single-dose vials.
VA Research Pharmacy will maintain complete, current records showing receipt, dispensing, and returns or destruction of Study Drug as required by Eisai's written instructions, the Protocol, applicable federal, state and local laws, regulations and guidelines.

VA Research Pharmacy will return to Eisai all unused Study Drug supplied by Eisai at the conclusion or early termination of the Study, or with prior written approval of Eisai, destroy all unused Study Drug and will provide certification to Eisai of such destruction. Sponsor Investigator or designated study monitor will review and approve VA Research Pharmacy reconciliation and destruction records before such records are submitted to Eisai.

5.8. Blinding of Study Drug

VA Research Pharmacy will be responsible for receipt, storage, dispensing, blinding/unblinding and destruction of Study Drug.

5.8.1. Receipt of Drug Supplies

Any damaged or unusable study drug in a given shipment will be documented by the VA Research Pharmacy. The Research Pharmacist will notify the Sponsor Investigator and Eisai of any damaged study drug.

Admixture incompatibility
The diluted drug product is administered intravenously. Because of the physico-chemical properties of eritoran, micelles (*i.e.*, small aggregates) are formed in the reconstituted and admixed solutions. Since the micelle size might affect the disposition of eritoran after intravenous administration, the micelle size is controlled through the manufacture and formulation of the drug product. Specific admixing instructions are as follows:

- Do not dilute or reconstitute eritoran with saline solutions or mix with other drugs or electrolytes.
- The use of any solution other than those recommended may cause micelle size changes of eritoran, and may affect its disposition.
- If administered through an existing intravenous line, flush with 5% Dextrose Injection, USP before and after infusion.
- Chlorhexidine-treated lumens may cause precipitation and an increase in micelle size and may affect the rate of clearance from plasma.
- Eritoran should only be admixed with 50 mL or 100 mL 5% Dextrose solution (D5W). Eritoran should not be admixed with other drugs.

5.8.2. Storage

The drug product used for clinical studies has a shelf-life of 5 years. The reconstituted drug product is

stable during storage at room temperature (or refrigerated) for up to 24 hours.

Eritoran clinical trial supplies will be kept in the original packaging and stored in a secure location at a temperature between 15-30 °C (59 to 86 °F), protected from light. Additional site-specific guidelines for storage of investigational products, and any country-specific requirements specified in the product label must be followed.

After reconstitution and admixture, storage of solutions of Eritoran will be limited to 4 hours at 15-30°C or up to 24 hours at 2-8 °C (36.5 to 46.4°F). Do not freeze. **Reconstituted Eritoran not administered within 24 hours should be discarded.**

For more details, please see also Section 15, Attachment F., Investigator Brochure.

5.8.3. Dispensing of Study Drug

Designated staff from the Audie L. Murphy Hospital Research Pharmacy maintain the Drug Administration Logs to track how, when and to whom the investigational drug was dispensed and assigned to subjects. Study clinical staff will keep administration records regarding dosing, unused drug, drug damaged, or wasted.

Routine Study Drug reconciliation will be performed based on STVHCS Audie L. Murphy VA Hospital policy and standard operating procedures.

5.8.4. Return or Destruction of Study Drug

The procedures for final reconciliation of the site's drug supply at the end of the study will be in accordance with VA Research Pharmacy standard operating procedures.

There are no special precautions cited for disposal of eritoran for injection.

6. Study Procedures

Study visits. All studies will be performed in the clinical research center (named Bartter Research Unit; BRU) located in the Audie L. Murphy VA Hospital (part of the South Texas Veterans Health Care System) in San Antonio, TX.

6.1. Visit 1

Following an overnight fast (no food after 9 PM) subjects will come to the BRU at approximately 7 AM for consenting, followed by screening procedures including a medical history, physical exam, and screening tests (CBC, chemistry, lipids, HbA1c, PT, PTT, urinalysis and ECG). At the discretion of the PI, blood coagulation studies (platelets, PT, PTT) may be repeated every 4-6 months during the study.

6.2. Visit 2

Within 3- 45 days after Visit 1, eligible subjects will return to the BRU at 7 AM after an overnight fast for an oral glucose tolerance test (OGTT) and whole body DEXA. The DEXA is done to quantify fat and lean mass. Lean mass values are used to adjust measurements of insulin-mediated glucose disposal.

6.3. Visit 3 – Infusion Protocol

Visit 3: Within 3 - 45 days after Visit 2, subjects will return to the BRU for the first eritoran/vehicle infusion. Hematology, comprehensive metabolic panel, including liver function tests and lipid panel will be performed prior to the infusion at Visits 3 and 5 (on Days 1, 2 and 3). If LDL cholesterol

increases more than 10% from Day 1 to Day 3 (on Visit 3 or 5), lipids will be re-assessed on the following visit.

Schema of Visit 3. Subjects will receive either placebo (vehicle) or eritoran, randomly assigned. If on Visit 3 they received placebo, on Visit 6 they will receive eritoran, and vice versa.

For visit 3, subjects will be admitted at approximately 7AM on the day of the eritoran/vehicle infusion. At approximately ~10AM subjects will receive eritoran (or vehicle) through an antecubital vein at a dose of 12 mg every 12 h for 3 days (72 mg total). The half-lives of LPS (~5 min) (61) and FFA (~2-4 min) (62) are very short. Thus, blocking TLR4 for 72 h will be sufficient to protect the receptor from being activated by these agonists. While in the BRU, subjects are ambulatory and fed 3 times a day with an isocaloric diet based on their habitual energy intake and food preferences. 68 h after the first eritoran/vehicle dose, a 2-step euglycemic hyperinsulinemic clamp (63) with 3-^{3}H glucose is started (at ~5-6 AM). During Step 1 (0-120 min) insulin is infused at 20 mU/m^2.min rate to measure hepatic and muscle insulin sensitivity. During Step 2 (120-240 min) insulin is infused at a 80 mU/m^2.min rate to measure peripheral (muscle) insulin sensitivity (63). Indirect calorimetry is performed for 30 min immediately before the insulin clamp and at the end of each clamp step. A subcutaneous adipose tissue biopsy (64) and a *vastus lateralis* muscle needle biopsy (65) will be performed 45 and 30 min before the start of the insulin clamp, respectively. A second muscle biopsy will be performed on the contralateral leg at the end of the insulin clamp Subjects will be given lunch at ~1 PM, monitored for three hours, then discharged to home.

Rationale behind dose**.** Eritoran has a half-life of ~25-30 h and pharmacodynamic studies have shown that ~12 h after eritoran administration (12 mg), the inhibitory effect of the drug on TLR4 signaling is fully maintained (59). This dose (12 mg) is safe and well tolerated, and higher doses do not confer further inhibitory effect on TLR4 (59).

Analyses.

Muscle: On pre-clamp samples we will perform TLR4 signaling assays [TLR4 protein, NFκB and MAPK (JNK, p38, ERK) phosphorylation/activity; ~50 mg tissue], inflammatory gene (TLR4, IL-6, MCP-1, iNOS, TNFα) expression (~20 mg), and intramyocellular lipid (ceramide, diacylglycerol) content (~20 mg), as described by our group (25, 59, 65, 66). On pre-clamp and clamp samples we will perform insulin signaling assays (~30 mg) (67). On pre-clamp samples macrophage infiltration and inflammatory state will be assessed by immunohistochemistry (IHC) (68-70) and real-time PCR (71) using macrophage markers (IHC: CD68 for total, CD40 for M1 and CD206 for M2 macrophages; PCR: EMR-1).

Adipose tissue: We will perform assays of TLR4 signaling (TLR4, IKK/NFκB and MAPKs) (~200 mg tissue) and inflammatory gene (TLR4, IL-6, MCP-1, iNOS, TNFα) expression (~200 mg). Macrophage infiltration and inflammatory state will be assessed by IHC and FACS, as described by our group (72).

Blood/plasma: Insulin, FFA and tritiated glucose are assayed 18 times during the clamp. Plasma cytokines (Inflammation Panel; Myriad RBM), LPS and LBP are assayed before the first eritoran dose and just before the clamp. Plasma LBP is measured because it is considered an integrated measure of plasma LPS level and indicator of subclinical endotoxemia (73). Measurement of TLR4 expression and TLR4 signaling (MAPK and NFκB phosphorylation) in monocytes will be done on whole blood samples collected immediately before the first eritoran dose (Day 1) and just before the insulin clamp (Day 4). To confirm the inhibitory effect of eritoran on TLR4, an *ex vivo* LPS stimulation test will be performed on whole blood samples (59).

6.4. Visit 4

5-7 days after Visit 3 all subjects come to the BRU for inspection of the biopsy sites. Subjects will maintain an isocaloric diet and will refrain from regular exercise until the next infusion protocol. If the lipid levels increased >10% during the Visit 3 hospital stay (from Day 1 to Day 3 measurements), we may repeat lipids (LDL cholesterol) at Visit 4.

6.5. Visit 5

Approximately 4 - 10 weeks after Visit 4 all subjects come to the BRU to undergo the second infusion protocol (same as Visit 3).

6.6. Visit 6

5-7 days after Visit 5 all subjects come to the BRU for inspection of the biopsy sites. If a 10% increase in lipid levels was observed during the Visit 5 hospital stay (from Day 1 to Day 3), we may repeat the lipid levels (LDL cholesterol) on Visit 6.

7. Statistical Plan

7.1. Sample Size Determination

The primary hypothesis is that the TLR4 antagonist will increase insulin sensitivity, especially in the insulin resistant subjects (obese and T2DM). The effect of a TLR4 inhibitor on insulin sensitivity has never been tested in humans. Therefore, we conducted the sample size calculation based on data using acipimox, a drug which rapidly improves insulin sensitivity by reducing the plasma level of FFA (a TLR4 ligand). In prior studies, the effect of acipimox on insulin sensitivity in T2DM subjects was a mean increase in Rd of 2.2 mg/kg.min with a SD of 3.46 (67). Based on this, the effect size would be approximately 0.64 assuming a within subject correlation of 0.5.

Hence with n=20 completers per each group (lean, obese nondiabetic, obese T2DM) and an effect size of 0.64, a paired t-test of the within subject difference in insulin sensitivity (between the vehicle and the TLR4 inhibitor) would have a power of 77% with a two-sided α of 0.05. We will enroll ~90 subjects to account for a ~30% dropout rate.

7.2. Statistical Methods

Analytical Approach. The goal of the analysis is to identify treatment-related changes in insulin sensitivity. With this crossover design, we will compare means between treatment groups using a repeated-measures General Linear Model (GML) analysis. This is expected to yield better statistical power than the use of the paired t-test used for the power calculation. We will determine the relationship between measurements of inflammation (TLR4 signaling, inflammatory genes) vs. insulin signaling/sensitivity using this regression approach as well as model the effect of selected covariates (e.g., sex and race/ethnicity). Scatter plots will be generated to identify outliers and to verify statistical assumptions.

8. Safety and Adverse Events

8.1. Definitions

Unanticipated Problems Involving Risk to Subjects or Others (UPIRSO)
Any incident, experience, or outcome that meets all of the following criteria:

- Unexpected in nature, severity, or frequency (i.e. not described in study-related documents such as the IRB-approved protocol or consent form, the investigators brochure, etc)
- Related or possibly related to participation in the research (i.e. possibly related means there is a reasonable possibility that the incident experience, or outcome may have been caused by the procedures involved in the research)
- Suggests that the research places subjects or others at greater risk of harm (including physical, psychological, economic, or social harm).

Adverse Event (AE)
In general, AE is used very broadly and encompasses physical and psychological harms and includes:

> Any untoward or unfavorable medical occurrence in a human subject, including any abnormal sign (for example, abnormal physical exam or laboratory finding), symptom, or disease, temporally associated with the subject's participation in the research, whether or not it is considered related to the subject's participation in the research.

Intercurrent illnesses or injuries should be regarded as adverse events. Abnormal results of diagnostic procedures are considered to be adverse events if the abnormality:

- results in study withdrawal
- is associated with a serious adverse event
- is associated with clinical signs or symptoms
- leads to additional treatment or to further diagnostic tests
- is considered by the investigator to be of clinical significance

Serious Adverse Event (SAE)
Adverse events are classified as serious or non-serious. A serious adverse event is any AE that:

- results in death;
- is life-threatening (places the subject at immediate risk of death from the event as it occurred);
- results in inpatient hospitalization or prolongation of existing hospitalization;
- results in a persistent or significant disability/incapacity;

- results in a congenital anomaly/birth defect; or
- based upon appropriate medical judgment, may jeopardize the subject's health and may require medical or surgical intervention to prevent one of the other outcomes listed in this definition (examples of such events include allergic bronchospasm requiring intensive treatment in the emergency room or at home, blood dyscrasias or convulsions that do not result in inpatient hospitalization, or the development of drug dependency or drug abuse).

Adverse Event Reporting Period
The study period during which adverse events must be reported is normally defined as the period from the initiation of any study procedures to the end of the study treatment follow-up. For this study, the study treatment follow-up is defined as 30 days following the last administration of study treatment.

Pre-existing Condition
A preexisting condition is one that is present at the start of the study. A pre-existing condition should be recorded as an adverse event if the frequency, intensity, or the character of the condition worsens during the study period.

General Physical Examination Findings
At screening, any clinically significant abnormality should be recorded as a preexisting condition. At the end of the study, any new clinically significant findings/abnormalities that meet the definition of an adverse event must also be recorded and documented as an adverse event.

Abnormal Laboratory Values
A clinical laboratory abnormality should be documented as an adverse event if any one of the following conditions is met:

- The laboratory abnormality is not otherwise refuted by a repeat test to confirm the abnormality
- The abnormality suggests a disease and/or organ toxicity
- The abnormality is of a degree that requires active management; e.g. change of dose, discontinuation of the drug, more frequent follow-up assessments, further diagnostic investigation, etc.

Hospitalization, Prolonged Hospitalization or Surgery
Any adverse event that results in hospitalization or prolonged hospitalization should be documented and reported as a SAE unless specifically instructed otherwise in this protocol. Any condition responsible for surgery should be documented as an AE if the condition meets the criteria for and adverse event.

Neither the condition, hospitalization, prolonged hospitalization, nor surgery are reported as an AE in the following circumstances:

- Hospitalization or prolonged hospitalization for diagnostic or elective surgical procedures for a preexisting condition.
- Surgery should not be reported as an outcome of an adverse event if the purpose of the surgery was elective or diagnostic and the outcome was uneventful.
- Hospitalization or prolonged hospitalization required to allow efficacy measurement for the study.
- Hospitalization or prolonged hospitalization for therapy of the target disease of the study, unless it is a worsening or increase in frequency of hospital admissions as judged by the clinical investigator.

8.2. Recording of Adverse Events

At each contact with the subject, the investigator or study staff will seek information about adverse events by specific questioning and, if appropriate, by examination. Information on all AEs will be recorded immediately in the source document, and also in the appropriate AE section of the case report

form (CRF). AEs will be tracked using the HSC IRB AE tracking form or data management tool (See Section 9.3) to be reviewed by Sponsor Investigator on a monthly and ad hoc basis, depending on severity and expected/unexpected nature of the event. A summary of AEs tracked will be reported in the quarterly report and cumulatively in the annual reports to Eisai and FDA.

All AEs occurring during the study period will be recorded. The clinical course of each event will be followed until resolution, stabilization, or until it has been determined that the study treatment or participation is not the cause. SAEs that are still ongoing at the end of the study period will be followed up to determine the final outcome. Any SAE that occurs after the study period and is considered to be possibly related to the study treatment or study participation will be recorded and reported per Institutional policy and according to FDA requirements.

8.3. Reporting of Serious Adverse Events and Unanticipated Problems

SAEs and UPIRSOs will be reported per IRB policy and procedure and in accordance with Eisai Inc., Material Transfer Agreement (See Section 15, Attachment A, Material Transfer Agreement, pages 18-21).

Each subject is evaluated for any adverse events. Any event that is reported to either the principal investigator or designated research associates by the subject or medical staff caring for the subject and which meets the criteria will be documented as such. Any serious event that is reported will then generate an adverse event report, which will be submitted to the IRB, R&D Committee, and the designated safety officer.

Unanticipated risks to subjects or others (UPIRSO) that are a result of study participation are promptly reported to the IRB, the R&D Committee, and the designated safety officer. The report will include a description of the event, when and how it was reported, as well as any official chart records or documentation to corroborate the event or the reporting of the event. All adverse events will be graded as mild, moderate, or severe. All adverse events will be summarized annually and submitted to the IRB and R&D Committees. Any action resulting in a temporary or permanent suspension of this study (e.g. IRB actions, R&D actions or actions by Eisai Inc.) will be reported to the appropriate VA and NIH program official.

8.3.1. Investigator reporting: notifying the study sponsor

The Sponsor Investigator will comply with all safety reporting regulations as set forth in the Code of Federal Regulations. Any correspondence to the FDA regarding adverse events or other safety issues will be simultaneously copied via facsimile or email to Eisai.

Institution and Sponsor Investigator shall cooperate with Eisai in all of their efforts to capture information on the safety profile of the Study Drug, including, but not limited to the following:

> Notifying Eisai of all serious suspected adverse drug reactions occurring in an individual who has been exposed to the Study Drug, and where the Study Drug is the suspected product.
>
> Where adverse drug reaction is defined as: a noxious and unintended response to a medicinal product related to any dose. A causal relationship between the medicinal product and the adverse response is at least a reasonable possibility. Serious is defined in Section 8.1, Definitions, see SAE.

A study-related UPIRSO, involving any type of SAE, must be reported to Eisai Inc. by telephone within 24 hours of the event. To report such events, a Serious Adverse Event (SAE) form must be completed by the investigator and faxed to the study sponsor within 24 hours, using the SAE form provided in the Material Transfer Agreement, Attachment B, and also referenced in Attachment B Section 15. The investigator will keep a copy of this SAE form on file at the study site.

Report SAEs by email and facsimile to:

Sally T. Ishizaka, Ph.D.
Sally_Ishizaka@eisai.com
Director of Biology
CINO Group, EISAI Inc.
Andover, MA 01810
978-837-4642

Within the following 48 hours, the investigator must provide further information on the SAE or the UPIRSO in the form of a written narrative. This should include a copy of the completed SAE form, and any other diagnostic information that will assist the understanding of the event. Significant new information on ongoing serious adverse events should be provided promptly to Eisai, Inc. A summary of non-serious AEs will be reported in the quarterly and annual reports to Eisai, Inc., and in the annual report to FDA.

8.3.2. Investigator reporting: notifying the UTHSCSA IRB

Reporting Process
All adverse events will be summarized annually and submitted to the IRB and R&D Committees. Any action resulting in a temporary or permanent suspension of this study (e.g. IRB actions, R&D actions or actions by the sponsor) will be reported to the appropriate VA program official.

Unanticipated problems posing risks to subjects or others as noted above will be reported to the UTHSCSA IRB using the form: UPIRSO Reporting Form or as a written report of the event (including a description of the event with information regarding its fulfillment of the above criteria, follow-up/resolution and need for revision to consent form and/or other study documentation).

Copies of each report and documentation of IRB notification and receipt will be kept in the study file.

8.3.3. Sponsor reporting: Notifying the FDA

The Sponsor Investigator is required to report certain study events to Eisai, Inc. thereby enabling the company providing the Study Drug whose IND is cross-referenced in Dr. Musi's IND application to report in an expedited fashion to the FDA in an IND safety report. The following describes the safety reporting requirements by timeline for reporting an associated type of event:

- ***Within 7 calendar days***
  Any study event that is:
  - associated with the use of the study drug
  - unexpected,
  - fatal or life-threatening, and

- ***Within 15 calendar days***
  Any study event that is:
  - associated with the use of the study drug,
  - unexpected, and
  - serious, but not fatal or life-threatening

    -or-
  - a previous adverse event that was not initially deemed reportable but is later found to fit the criteria for reporting (reporting within 15 calendar days from when event was deemed reportable).

  Any finding from tests in laboratory animals that:
  - suggests a significant risk for human subjects including reports of mutagenicity, teratogenicity, or carcinogenicity.

Additional reporting requirements
Eisai Inc. is also required to identify in IND safety reports all previous reports concerning similar adverse events and to analyze the significance of the current event in light of the previous reports.

Reporting Process
Adverse events may be submitted on FDA Form 3500A or in a narrative format. The contact information for submitting IND safety reports is noted below:

Food and Drug Administration

Center for Drug Evaluation and Research
Division of Metabolism and Endocrinology Products
5901-B Ammendale Road
Beltsville, MD 20705-1266
Phone: (301) 796-2290
Fax: (301) 796-9712

8.4. Medical Monitoring

The Sponsor Investigator will review the safety and progress of this study on a monthly basis or when needed if SAE or SAE-UPIRSO occurs. The Designated Safety Officer is Sandra Sanchez Reilly, M.D. Dr. Sanchez Reilly is a Professor in the Department of Medicine, UTHSCSA, and Staff Physician at the Audie L Murphy VA Hospital.

Twice a year the Sponsor Investigator (PI) will provide study safety information to the Designated Safety Officer (DSO) to conduct reviews of overall subject safety for the study. Within 30 days of receiving safety information for routine reviews, the Designated Safety Officer will provide the Sponsor Investigator a written synopsis of the review and identify safety concerns, if any, with a recommended plan of action. Ad hoc reviews in real-time may occur upon mutual agreement between Sponsor Investigator and Designated Safety Officer.

8.5. Unblinding Procedures

See Section 5.8 – If, in the case of SAE or SAE-UPIRSO, the Clinical Study Coordinator or appropriately designated study staff will contact the Designated Safety Officer (Section 8.6) to assess the need for initiation of unblinding procedures. Responsibilities of each individual are bulleted below.

8.5.1. Designated Safety Officer

- Assess SAE or SAE-UPIRSO to determine if unblinding is required and, if yes, notify pharmacist to request subject's assignment
- Compare event data to intervention/assignment to determine if SAE is related to study intervention
- Assess study and subject safety to determine if stopping rules should be invoked
- Create the Safety Officer record in a blinded report format and communicate to Sponsor Investigator and Clinical Study Coordinator with overall impression and recommended plan of action
- Responsible for determining the need to "unblind" the investigator

8.5.2. VA Research Pharmacist

- Unblind subject assignment communicated to Designated Safety Officer upon request
- Suspend dispensing of study drug until SAE reporting is complete

8.5.3. Sponsor Investigator

- Receive and review Designated Safety Officer report with Clinical Study Coordinator, Pharmacist and Study Statistician
- Determine and implement stopping rules, if required
- Ensure that IRB, FDA, Eisai and NIH are notified as appropriate to local or agency policy

8.5.4. Clinical Study Coordinator

- Obtain a copy of Designated Safety Officer report/record to file in study binder/file and forward a copy to the Regulatory Coordinator
- Ensure appropriate medical care and follow up is scheduled and implemented until SAE resolved
- Create FDA report (MedWatch), if applicable

8.6. Stopping Rules

Clinical or laboratory excursions that a subject may experience that may warrant stopping study participation or drug administration, either temporarily or permanently, include but are not limited to:

Clinical criteria:

- Fever with a temperature >101°F
- Allergic reaction to drug
- Blood pressure >160/95 or <80/50
- Severe vomiting
- Phlebitis grade 4 or 5 (see Attachment G.)

Laboratory criteria:

- Liver function test results >2x normal range
- WBC ≥14,000 or ≤3,000
- HCT ≥55 or ≤33
- Platelets ≤100,000 or ≥700,000

In the unlikely event that a study-related death or SAE occurs, the decision to stop the trial, either temporarily or permanently, will be the responsibility of the Designated Safety Officer in collaboration with the Sponsor Investigator.

9. Data Handling and Record Keeping

9.1. Confidentiality

Information learned about all subjects will be kept confidential. All data and protected health information in paper form will be kept confidential by assigned anonymous identifier and kept secured (password protected and/or double locked). Subjects will not be identified in any way in any publication.

9.2. Source Documents

Source data are contained in source documents found in paper subject files at the research site and in VA CPRS medical records. Print all entries legibly in blue or black ink. Erasures and white-out material are prohibited. If any entry error has been made on paper, to correct such an error, draw a single straight line through the incorrect entry and enter the correct data above it. All such changes must be initialed and dated. For clarification of illegible or uncertain entries, print the clarification above the item, then initial and date it.

9.3. Case Report Forms

The study case report forms (CRF) are the primary data collection instruments for the study. Data requested on the CRF will be collected from the subject encounter and from the subject diaries, pharmacy logs, and medical records (VA CPRS), then entered into the IDEAS database managed by the Department of Epidemiology and Biostatistics. All missing data will be routinely queried, corrected, and or explained. If a space on the CRF is left blank because the procedure was not done or the question was not asked, write “N/D”. If the item is not applicable to the individual case, write “N/A”.

9.4. Data Management

Database Management Software: All data management for this project will be maintained using the Informatics Data Exchange and Acquisition System (IDEAS) which was developed by the Department of Epidemiology and Biostatistics. The database engine consists of Oracle Enterprise which is maintained on a cluster of 22 Sun Microsystems servers. Database administration is performed via UNIX-based scripts, Oracle Enterprise Manager (OEM) and Tool for Oracle Application Developers (TOAD). The data system model is described below.

Data System: IDEAS is a computing environment consisting of a collection of applications, systems, policies, and procedures that govern its informatics operations. While the IDEAS integrates a variety of data from different sources, it was developed around support for clinical/translational research investigations. Starting with security and acting as an information broker facilitating data collection, analysis, and reporting, the system was designed to integrate disparate data from multiple and geographically separate collection and treatment facilities. Data applications are designed to be end-user oriented and constructed to optimize workflow and minimize errors. The IDEAS system capitalizes on its tiered architecture to implement numerous business rules and business objects to enforce a number of Quality Control (QC) procedures, IDEAS incorporates and utilizes pre-defined standardized Common Data Elements (CDEs), with the ability to incorporate new CDEs as they become available. It has been designed to follow the necessary standards and if needed, set the standards. IDEAS server resources are divided into four primary categories: 1) informatics and statistical computing; 2) data collection and reporting; 3) software development; and 4) infrastructure: file and naming services. This system has been in use for 5 years and has proven to be flexible, scalable and easily extendible to meet research, production, regulatory, reporting, and analytical needs. IDEAS modules support clinical, specimen, pathological, personnel, and project tracking applications. In addition to data collection and analysis, IDEAS facilitates performance monitoring and the enforcement of all human subject requirements (both local as well as Federal). IDEAS is the system used by our larger CTSA projects as well as by our National Cancer Institute P30-funded cancer center. We will utilize IDEAS to support all data management.

All data will be input using a web front-end interface. All users are individually assigned authorization for access to specific components of the database application. Information that is input is checked for logical and range consistency and mandatory data fields must be entered in order to input a record. We use a formal revision tracking procedure where all SAS code is documented by the analyst, giving the date, version and purpose. All database tables are fully labeled. Common field formats, such as those for sex and ethnicity, are used for all data applications. For analysis, al project data are directly accessible via direct SQL calls within SAS to Oracle (using PROC SQL). Reports are available using both Microsoft Word and Adobe pdf documents and automated tables and graphics generated using the SAS Output Delivery System (ODS).

9.5. Records Retention

The NIH Principal Investigator is responsible for maintaining study essential documents for at least 3 years after the NIH grant period ends or at least 2 years have elapsed since the formal discontinuation of clinical development of the investigational product, whichever is longer. These documents should be retained for a longer period if required by an agreement with the sponsor. In such an instance, it is the responsibility of the sponsor to inform the investigator/institution as to when these documents no longer need to be retained.

10. Study Monitoring, Auditing, and Inspecting

10.1. Study Monitoring Plan

The Sponsor Investigator will ensure that the designated study monitor, compliance specialist or other quality assurance reviewer is given access to all the above noted study-related documents and study

related facilities (e.g. pharmacy, diagnostic laboratory, etc.), and has adequate space to conduct the study monitoring visits.

Monitoring elements are described below:

1. The quality and content of the informed consent, and the consent process will be monitored throughout the study period.
2. The accuracy of the collection and processing of laboratory specimens will be monitored throughout the study period.
3. The timeliness and conformity with established clinical monitoring of the subjects will be followed on a quarterly basis.
4. The forms and source documents in the subjects' research records will be current and maintained in a timely manner.
5. The physical and mental condition of the subject and any type of AE will be assessed and monitored prior to beginning the study by the investigator(s) and staff, throughout the study period.

Areas of focus for study monitoring by the designated study monitor:

1. Regulatory documentation and compliance with local policy will be monitored quarterly and throughout the study period.
2. The safe handling and accountability of drugs, which includes the use of protective equipment and disposal of waste, will be maintained prior to the start of the study and semi-annually throughout the study period.
3. Compliance with the protocol and conduct of the study, including the consenting process, along with source data compared to collected data (SDV), will be monitored on a quarterly basis throughout the study period.
    a. 50% of subject records and 20% of data for each subject will be monitored.
    b. The first and the last subject enrolled will be monitored for consent practices and 100% of data collected.
4. Adverse event logs will be monitored quarterly and research records analyzed for AEs that were overlooked or otherwise not documented properly in the study files.
5. The condition of the physical space used by the investigator and subject for study purposes will be monitored prior to the start of the study and annually throughout the study period.
6. The safety assurance and condition of any electrical, radiological, magnetic, gaseous, and hazardous equipment, tools or apparatus that will be used by the investigators and subjects will be monitored prior to the start of the study and annually throughout the study period.
7. The physical safety and security of the staff prior to the start of the study and throughout the study period will be maintained and monitored annually prior to continuing review.

10.2. Auditing and Inspecting

The Sponsor Investigator will permit study-related monitoring, audits, and inspections by the IRB, the funding sponsor, government regulatory bodies, and University compliance groups of all study related documents (e.g. source documents, regulatory documents, data collection instruments, study data etc.). The investigator will ensure the capability for inspections of applicable study-related facilities (e.g. pharmacy, diagnostic laboratory, etc.).

Participation as an investigator in this study implies acceptance of potential inspection by government regulatory authorities and applicable University compliance and quality assurance offices.

11. Ethical Considerations

This study is to be conducted according to US and international standards of Good Clinical Practice (FDA Title 21 part 312) applicable government regulations and Institutional research policies and procedures.

This protocol and any amendments will be submitted to the Institutional Review Board (IRB), in

agreement with local legal prescriptions, for formal approval of the study conduct. The decision of the IRB concerning the conduct of the study will be made in writing to the investigator and a copy of this decision will be provided to the funding sponsor before commencement of this study.

All subjects for this study will be provided a consent form describing this study and providing sufficient information for subjects to make an informed decision about their participation in this study. See Attachments, Section 15, Attachment D., Sample Informed Consent Form. This consent form will be submitted with the protocol for review and approval by the IRB for the study. The formal consent of a subject, using the IRB-approved consent form, must be obtained before that subject undergoes any study procedure. The consent form must be signed by the subject or legally authorized representative, and the investigator-designated research professional obtaining the consent.

12. Study Finances

12.1. Funding Source

This study is financed through a grant from the US National Institutes of Health/National Institute of Diabetes and Digestive and Kidney Diseases (NIH-NIDDK), R01DK080157.

12.2. Conflict of Interest

None reported.

12.3. Subject Stipends or Payments

This study will reimburse subjects. A schedule of payments is shown below. The total potential reimbursement to a subject is $1,680 for the study for all visits, or payments may be prorated to include the last visit completed if study participation is terminated early. Manual payments for additional visits, if necessary, will be handled on an ad-hoc basis with prior approval from the Sponsor Investigator.

Visit 1	$10
Visit 2	$50
Visit 3	$800
Visit 4	$10
Visit 5	$800
Visit 6	$10
Unscheduled Visit	Up to $100

13. Publication Plan

The Institution, Sponsor Investigator or respective designees may present or publish the results of a scientific investigation involving this Study in accordance with Attachment A., Material Transfer Agreement, Article 7., Publication/Release of Information.

14. References

See also References in Attachments, Section 15.

15. Attachments

A. EISAI Inc - Material Transfer Agreement (signed May 20, 2014)
B. EISAI Inc - Serious Adverse Event Reporting Form (Attachment to MTA)
C. Study Event Schedule
D. Sample Consent Form

E. Reference List
F. EISAI Inc - Investigator Brochure
G. Grading Scale for Phlebitis
H. Summary of Protocol Changes

ATTACHMENT A. MATERIAL TRANSFER AGREEMENT

ATTACHMENT B. EISAI, INC., SERIOUS ADVERSE EVENT REPORTING FORM

ATTACHMENT C. STUDY EVENT SCHEDULE

ATTACHMENT D. SAMPLE INFORMED CONSENT

ATTACHMENT E. REFERENCES

1. Cusi K, Maezono K, Osman A, Pendergrass M, Patti ME, Pratipanawatr T, et al. Insulin resistance differentially affects the PI 3-kinase- and MAP kinase-mediated signaling in human muscle. J Clin Invest. 2000;105(3):311-20. PubMed PMID: 10675357.
2. Karlsson HK, Zierath JR, Kane S, Krook A, Lienhard GE, Wallberg-Henriksson H. Insulin-stimulated phosphorylation of the Akt substrate AS160 is impaired in skeletal muscle of type 2 diabetic subjects. Diabetes. 2005;54(6):1692-7. Epub 2005/05/28. doi: 54/6/1692 [pii]. PubMed PMID: 15919790.
3. Zierath JR, Krook A, Wallberg-Henriksson H. Insulin action in skeletal muscle from patients with NIDDM. Mol Cell Biochem. 1998;182(1-2):153-60. PubMed PMID: 9609124.
4. DeFronzo RA, Ferrannini E, Simonson DC. Fasting hyperglycemia in non-insulin-dependent diabetes mellitus: contributions of excessive hepatic glucose production and impaired tissue glucose uptake. Metabolism. 1989;38(4):387-95. Epub 1989/04/01. PubMed PMID: 2657323.
5. Medzhitov R. Toll-like receptors and innate immunity. Nat Rev Immunol. 2001;1(2):135-45. PubMed PMID: 11905821.
6. Raetz CR. Biochemistry of endotoxins. Annu Rev Biochem. 1990;59:129-70. PubMed PMID: 1695830.
7. Lee JY, Sohn KH, Rhee SH, Hwang D. Saturated fatty acids, but not unsaturated fatty acids, induce the expression of cyclooxygenase-2 mediated through Toll-like receptor 4. J Biol Chem. 2001;276(20):16683-9. Epub 2001/03/30. doi: 10.1074/jbc.M011695200
M011695200 [pii]. PubMed PMID: 11278967.
8. Lee JY, Ye J, Gao Z, Youn HS, Lee WH, Zhao L, et al. Reciprocal modulation of Toll-like receptor-4 signaling pathways involving MyD88 and phosphatidylinositol 3-kinase/AKT by saturated and polyunsaturated fatty acids. J Biol Chem. 2003;278(39):37041-51. Epub 2003/07/17. doi: 10.1074/jbc.M305213200
M305213200 [pii]. PubMed PMID: 12865424.
9. Munford RS, Hall CL. Detoxification of bacterial lipopolysaccharides (endotoxins) by a human neutrophil enzyme. Science. 1986;234(4773):203-5. PubMed PMID: 3529396.
10. Muzio M, Ni J, Feng P, Dixit VM. IRAK (Pelle) family member IRAK-2 and MyD88 as proximal mediators of IL-1 signaling. Science. 1997;278(5343):1612-5. PubMed PMID: 9374458.
11. Aguirre V, Uchida T, Yenush L, Davis R, White MF. The c-Jun NH(2)-terminal kinase promotes insulin resistance during association with insulin receptor substrate-1 and phosphorylation of Ser(307). J Biol Chem. 2000;275(12):9047-54. PubMed PMID: 10722755.
12. Gao Z, Hwang D, Bataille F, Lefevre M, York D, Quon MJ, et al. Serine phosphorylation of insulin receptor substrate 1 by inhibitor kappa B kinase complex. J Biol Chem. 2002;277(50):48115-21. PubMed PMID: 12351658.
13. Evans JL, Goldfine ID, Maddux BA, Grodsky GM. Oxidative stress and stress-activated signaling pathways: a unifying hypothesis of type 2 diabetes. Endocr Rev. 2002;23(5):599-622. PubMed PMID: 12372842.
14. Yuan M, Konstantopoulos N, Lee J, Hansen L, Li ZW, Karin M, et al. Reversal of obesity- and diet-induced insulin resistance with salicylates or targeted disruption of Ikkbeta. Science. 2001;293(5535):1673-7. PubMed PMID: 11533494.
15. Kim JK, Kim YJ, Fillmore JJ, Chen Y, Moore I, Lee J, et al. Prevention of fat-induced insulin resistance by salicylate. J Clin Invest. 2001;108(3):437-46. PubMed PMID: 11489937.
16. Hirosumi J, Tuncman G, Chang L, Gorgun CZ, Uysal KT, Maeda K, et al. A central role for JNK in obesity and insulin resistance. Nature. 2002;420(6913):333-6. PubMed PMID: 12447443.
17. Reaven GM, Chen YD. Role of abnormal free fatty acid metabolism in the development of non-insulin-dependent diabetes mellitus. Am J Med. 1988;85(5A):106-12. PubMed PMID: 3057887.
18. Santomauro AT, Boden G, Silva ME, Rocha DM, Santos RF, Ursich MJ, et al. Overnight lowering of free fatty acids with Acipimox improves insulin resistance and glucose tolerance in obese diabetic and

nondiabetic subjects. Diabetes. 1999;48(9):1836-41. Epub 1999/09/10. PubMed PMID: 10480616.
19. Bajaj M, Suraamornkul S, Kashyap S, Cusi K, Mandarino L, DeFronzo RA. Sustained reduction in plasma free fatty acid concentration improves insulin action without altering plasma adipocytokine levels in subjects with strong family history of type 2 diabetes. J Clin Endocrinol Metab. 2004;89(9):4649-55. PubMed PMID: 15356076.
20. Belfort R, Mandarino L, Kashyap S, Wirfel K, Pratipanawatr T, Berria R, et al. Dose-response effect of elevated plasma free fatty acid on insulin signaling. Diabetes. 2005;54(6):1640-8. Epub 2005/05/28. doi: 54/6/1640 [pii]. PubMed PMID: 15919784.
21. Phielix E, Meex R, Ouwens DM, Sparks L, Hoeks J, Schaart G, et al. High oxidative capacity due to chronic exercise training attenuates lipid-induced insulin resistance. Diabetes. 2012;61(10):2472-8. Epub 2012/07/13. doi: db11-1832 [pii]
10.2337/db11-1832. PubMed PMID: 22787138; PubMed Central PMCID: PMC3447923.
22. Lee JY, Zhao L, Youn HS, Weatherill AR, Tapping R, Feng L, et al. Saturated fatty acid activates but polyunsaturated fatty acid inhibits Toll-like receptor 2 dimerized with Toll-like receptor 6 or 1. J Biol Chem. 2004;279(17):16971-9. PubMed PMID: 14966134.
23. Hussey SE, Liang H, Costford SR, Klip A, DeFronzo RA, Sanchez-Avila A, et al. TAK-242, a small-molecule inhibitor of Toll-like receptor 4 signalling, unveils similarities and differences in lipopolysaccharide- and lipid-induced inflammation and insulin resistance in muscle cells. Biosci Rep. 2013;33(1):37-47. Epub 2012/10/12. doi: BSR20120098 [pii]
10.1042/BSR20120098. PubMed PMID: 23050932; PubMed Central PMCID: PMC3522475.
24. Radin MS, Sinha S, Bhatt BA, Dedousis N, O'Doherty RM. Inhibition or deletion of the lipopolysaccharide receptor Toll-like receptor-4 confers partial protection against lipid-induced insulin resistance in rodent skeletal muscle. Diabetologia. 2008;51(2):336-46. Epub 2007/12/07. doi: 10.1007/s00125-007-0861-3. PubMed PMID: 18060381.
25. Reyna SM, Ghosh S, Tantiwong P, Meka CS, Eagan P, Jenkinson CP, et al. Elevated toll-like receptor 4 expression and signaling in muscle from insulin-resistant subjects. Diabetes. 2008;57(10):2595-602. Epub 2008/07/18. doi: db08-0038 [pii]
10.2337/db08-0038. PubMed PMID: 18633101; PubMed Central PMCID: PMC2551667.
26. Shi H, Kokoeva MV, Inouye K, Tzameli I, Yin H, Flier JS. TLR4 links innate immunity and fatty acid-induced insulin resistance. J Clin Invest. 2006;116(11):3015-25. Epub 2006/10/21. doi: 10.1172/JCI28898. PubMed PMID: 17053832; PubMed Central PMCID: PMC1616196.
27. Holland WL, Bikman BT, Wang LP, Yuguang G, Sargent KM, Bulchand S, et al. Lipid-induced insulin resistance mediated by the proinflammatory receptor TLR4 requires saturated fatty acid-induced ceramide biosynthesis in mice. J Clin Invest. 2011;121(5):1858-70. Epub 2011/04/15. doi: 43378 [pii]
10.1172/JCI43378. PubMed PMID: 21490391.
28. Tsukumo DM, Carvalho-Filho MA, Carvalheira JB, Prada PO, Hirabara SM, Schenka AA, et al. Loss-of-function mutation in Toll-like receptor 4 prevents diet-induced obesity and insulin resistance. Diabetes. 2007;56(8):1986-98. Epub 2007/05/24. doi: db06-1595 [pii]
10.2337/db06-1595. PubMed PMID: 17519423.
29. Galbo T, Perry RJ, Jurczak MJ, Camporez JP, Alves TC, Kahn M, et al. Saturated and unsaturated fat induce hepatic insulin resistance independently of TLR-4 signaling and ceramide synthesis in vivo. Proc Natl Acad Sci U S A. 2013;110(31):12780-5. Epub 2013/07/11. doi: 1311176110 [pii]
10.1073/pnas.1311176110. PubMed PMID: 23840067; PubMed Central PMCID: PMC3732992.
30. Dasu MR, Devaraj S, Park S, Jialal I. Increased toll-like receptor (TLR) activation and TLR ligands in recently diagnosed type 2 diabetic subjects. Diabetes Care. 2010;33(4):861-8. Epub 2010/01/14. doi: dc09-1799 [pii]
10.2337/dc09-1799. PubMed PMID: 20067962; PubMed Central PMCID: PMC2845042.
31. Reyna SM, Tantiwong P, Cersosimo E, Defronzo RA, Sriwijitkamol A, Musi N. Short-term exercise training improves insulin sensitivity but does not inhibit inflammatory pathways in immune cells from insulin-resistant subjects. J Diabetes Res. 2013;2013:107805. Epub 2013/05/15. doi: 10.1155/2013/107805. PubMed PMID: 23671849; PubMed Central PMCID: PMC3647562.
32. Turnbaugh PJ, Ley RE, Hamady M, Fraser-Liggett CM, Knight R, Gordon JI. The human microbiome project. Nature. 2007;449(7164):804-10. Epub 2007/10/19. doi: nature06244 [pii]

10.1038/nature06244. PubMed PMID: 17943116.
33. Membrez M, Blancher F, Jaquet M, Bibiloni R, Cani PD, Burcelin RG, et al. Gut microbiota modulation with norfloxacin and ampicillin enhances glucose tolerance in mice. FASEB J. 2008;22(7):2416-26. Epub 2008/03/11. doi: fj.07-102723 [pii]
10.1096/fj.07-102723. PubMed PMID: 18326786.
34. Cani PD, Bibiloni R, Knauf C, Waget A, Neyrinck AM, Delzenne NM, et al. Changes in gut microbiota control metabolic endotoxemia-induced inflammation in high-fat diet-induced obesity and diabetes in mice. Diabetes. 2008;57(6):1470-81. Epub 2008/02/29. doi: db07-1403 [pii]
10.2337/db07-1403. PubMed PMID: 18305141.
35. Zaibi MS, Stocker CJ, O'Dowd J, Davies A, Bellahcene M, Cawthorne MA, et al. Roles of GPR41 and GPR43 in leptin secretory responses of murine adipocytes to short chain fatty acids. FEBS Lett. 2010;584(11):2381-6. Epub 2010/04/20. doi: S0014-5793(10)00297-8 [pii]
10.1016/j.febslet.2010.04.027. PubMed PMID: 20399779.
36. Ferraris RP, Vinnakota RR. Intestinal nutrient transport in genetically obese mice. Am J Clin Nutr. 1995;62(3):540-6. Epub 1995/09/01. PubMed PMID: 7661115.
37. Liang H, Hussey SE, Sanchez-Avila A, Tantiwong P, Musi N. Effect of lipopolysaccharide on inflammation and insulin action in human muscle. PLoS One. 2013;8(5):e63983. Epub 2013/05/25. doi: 10.1371/journal.pone.0063983
PONE-D-12-37717 [pii]. PubMed PMID: 23704966; PubMed Central PMCID: PMC3660322.
38. Creely SJ, McTernan PG, Kusminski CM, Fisher f M, Da Silva NF, Khanolkar M, et al. Lipopolysaccharide activates an innate immune system response in human adipose tissue in obesity and type 2 diabetes. Am J Physiol Endocrinol Metab. 2007;292(3):E740-7. Epub 2006/11/09. doi: 00302.2006 [pii]
10.1152/ajpendo.00302.2006. PubMed PMID: 17090751.
39. Al-Attas OS, Al-Daghri NM, Al-Rubeaan K, da Silva NF, Sabico SL, Kumar S, et al. Changes in endotoxin levels in T2DM subjects on anti-diabetic therapies. Cardiovasc Diabetol. 2009;8:20. Epub 2009/04/17. doi: 1475-2840-8-20 [pii]
10.1186/1475-2840-8-20. PubMed PMID: 19368716; PubMed Central PMCID: PMC2674418.
40. Opal SM, Scannon PJ, Vincent JL, White M, Carroll SF, Palardy JE, et al. Relationship between plasma levels of lipopolysaccharide (LPS) and LPS-binding protein in patients with severe sepsis and septic shock. J Infect Dis. 1999;180(5):1584-9. Epub 1999/10/09. doi: JID990373 [pii]
10.1086/315093. PubMed PMID: 10515819.
41. Ghanim H, Abuaysheh S, Sia CL, Korzeniewski K, Chaudhuri A, Fernandez-Real JM, et al. Increase in plasma endotoxin concentrations and the expression of Toll-like receptors and suppressor of cytokine signaling-3 in mononuclear cells after a high-fat, high-carbohydrate meal: implications for insulin resistance. Diabetes Care. 2009;32(12):2281-7. Epub 2009/09/17. doi: dc09-0979 [pii]
10.2337/dc09-0979. PubMed PMID: 19755625; PubMed Central PMCID: PMC2782991.
42. Deopurkar R, Ghanim H, Friedman J, Abuaysheh S, Sia CL, Mohanty P, et al. Differential effects of cream, glucose, and orange juice on inflammation, endotoxin, and the expression of Toll-like receptor-4 and suppressor of cytokine signaling-3. Diabetes Care. 2010;33(5):991-7. Epub 2010/01/14. doi: dc09-1630 [pii]
10.2337/dc09-1630. PubMed PMID: 20067961; PubMed Central PMCID: PMC2858203.
43. Amar J, Burcelin R, Ruidavets JB, Cani PD, Fauvel J, Alessi MC, et al. Energy intake is associated with endotoxemia in apparently healthy men. Am J Clin Nutr. 2008;87(5):1219-23. Epub 2008/05/13. doi: 87/5/1219 [pii]. PubMed PMID: 18469242.
44. Cani PD, Possemiers S, Van de Wiele T, Guiot Y, Everard A, Rottier O, et al. Changes in gut microbiota control inflammation in obese mice through a mechanism involving GLP-2-driven improvement of gut permeability. Gut. 2009;58(8):1091-103. Epub 2009/02/26. doi: gut.2008.165886 [pii]
10.1136/gut.2008.165886. PubMed PMID: 19240062.
45. Cani PD, Neyrinck AM, Fava F, Knauf C, Burcelin RG, Tuohy KM, et al. Selective increases of bifidobacteria in gut microflora improve high-fat-diet-induced diabetes in mice through a mechanism associated with endotoxaemia. Diabetologia. 2007;50(11):2374-83. Epub 2007/09/08. doi: 10.1007/s00125-007-0791-0. PubMed PMID: 17823788.

46. Ghoshal S, Witta J, Zhong J, de Villiers W, Eckhardt E. Chylomicrons promote intestinal absorption of lipopolysaccharides. J Lipid Res. 2009;50(1):90-7. Epub 2008/09/26. doi: M800156-JLR200 [pii]
10.1194/jlr.M800156-JLR200. PubMed PMID: 18815435.
47. Mehta NN, McGillicuddy FC, Anderson PD, Hinkle CC, Shah R, Pruscino L, et al. Experimental endotoxemia induces adipose inflammation and insulin resistance in humans. Diabetes. 2010;59(1):172-81. Epub 2009/10/02. doi: db09-0367 [pii]
10.2337/db09-0367. PubMed PMID: 19794059.
48. Pussinen PJ, Havulinna AS, Lehto M, Sundvall J, Salomaa V. Endotoxemia is associated with an increased risk of incident diabetes. Diabetes Care. 2011;34(2):392-7. Epub 2011/01/29. doi: 34/2/392 [pii]
10.2337/dc10-1676. PubMed PMID: 21270197; PubMed Central PMCID: PMC3024355.
49. Lassenius MI, Pietilainen KH, Kaartinen K, Pussinen PJ, Syrjanen J, Forsblom C, et al. Bacterial endotoxin activity in human serum is associated with dyslipidemia, insulin resistance, obesity, and chronic inflammation. Diabetes Care. 2011;34(8):1809-15. Epub 2011/06/04. doi: dc10-2197 [pii]
10.2337/dc10-2197. PubMed PMID: 21636801; PubMed Central PMCID: PMC3142060.
50. Hussey SE, Lum H, Alvarez A, Cipriani Y, Garduno J, Anaya L, et al. A sustained increase in plasma NEFA upregulates the toll-like receptor network in human muscle. Diabetologia. In press.
51. Muscogiuri G, Kamat A, Balas B, Giaccari A, Defronzo RA, Musi N, et al. beta-Adrenergic Responsive Induction of Insulin Resistance in Liver of Aging Rats. Endocr Res. 2011;36(2):74-82. Epub 2011/03/29. doi: 10.3109/07435800.2010.539993. PubMed PMID: 21438725.
52. Ehrentraut S, Lohner R, Schwederski M, Ehrentraut H, Boehm O, Noga S, et al. In vivo Toll-like receptor 4 antagonism restores cardiac function during endotoxemia. Shock. 2011;36(6):613-20. Epub 2011/11/18. doi: 10.1097/SHK.0b013e318235805f
00024382-201112000-00013 [pii]. PubMed PMID: 22089127.
53. Mullarkey M, Rose JR, Bristol J, Kawata T, Kimura A, Kobayashi S, et al. Inhibition of endotoxin response by eritoran, a novel Toll-like receptor 4-directed endotoxin antagonist. J Pharmacol Exp Ther. 2003;304(3):1093-102. Epub 2003/02/27. doi: 10.1124/jpet.102.044487. PubMed PMID: 12604686.
54. Tidswell M, Tillis W, Larosa SP, Lynn M, Wittek AE, Kao R, et al. Phase 2 trial of eritoran tetrasodium (ERITORAN), a toll-like receptor 4 antagonist, in patients with severe sepsis. Crit Care Med. 2010;38(1):72-83. Epub 2009/08/08. doi: 10.1097/CCM.0b013e3181b07b78. PubMed PMID: 19661804.
55. Wong YN, Rossignol D, Rose JR, Kao R, Carter A, Lynn M. Safety, pharmacokinetics, and pharmacodynamics of ERITORAN, a lipid A antagonist, during an ascending single-dose clinical study. J Clin Pharmacol. 2003;43(7):735-42. Epub 2003/07/15. PubMed PMID: 12856387.
56. Liang E, Wong YN, Allen I, Kao R, Marino M, DiLea C. Pharmacokinetics of ERITORAN, a lipopolysaccharide antagonist, in patients with impaired hepatic function. J Clin Pharmacol. 2003;43(12):1361-9. Epub 2003/11/15. doi: 10.1177/0091270003258653
43/12/1361 [pii]. PubMed PMID: 14615472.
57. Lynn M, Rossignol DP, Wheeler JL, Kao RJ, Perdomo CA, Noveck R, et al. Blocking of responses to endotoxin by ERITORAN in healthy volunteers with experimental endotoxemia. J Infect Dis. 2003;187(4):631-9. Epub 2003/02/25. doi: JID020976 [pii]
10.1086/367990. PubMed PMID: 12599080.
58. Lynn M, Wong YN, Wheeler JL, Kao RJ, Perdomo CA, Noveck R, et al. Extended in vivo pharmacodynamic activity of ERITORAN in normal volunteers with experimental endotoxemia [corrected]. J Pharmacol Exp Ther. 2004;308(1):175-81. Epub 2003/10/21. doi: 10.1124/jpet.103.056531
jpet.103.056531 [pii]. PubMed PMID: 14566003.
59. Rossignol DP, Wong N, Noveck R, Lynn M. Continuous pharmacodynamic activity of eritoran tetrasodium, a TLR4 antagonist, during intermittent intravenous infusion into normal volunteers. Innate Immun. 2008;14(6):383-94. Epub 2008/11/29. doi: 14/6/383 [pii]
10.1177/1753425908099173. PubMed PMID: 19039062.
60. Rossignol DP, Wasan KM, Choo E, Yau E, Wong N, Rose J, et al. Safety, pharmacokinetics, pharmacodynamics, and plasma lipoprotein distribution of eritoran (ERITORAN) during continuous intravenous infusion into healthy volunteers. Antimicrob Agents Chemother. 2004;48(9):3233-40. Epub 2004/08/26. doi: 10.1128/AAC.48.9.3233-3240.2004
48/9/3233 [pii]. PubMed PMID: 15328078; PubMed Central PMCID: PMC514793.

61. Beck-Nielsen H, Nielsen OH, Damsbo P, Vaag A, Handberg A, Skott P, et al. Insulin resistance: a risk factor for diabetic complications. J Diabet Complications. 1990;4(2):46-8. Epub 1990/04/01. PubMed PMID: 2145297.
62. Eaton RP, Berman M, Steinberg D. Kinetic studies of plasma free fatty acid and triglyceride metabolism in man. J Clin Invest. 1969;48(8):1560-79. Epub 1969/08/01. doi: 10.1172/JCI106122. PubMed PMID: 5796365; PubMed Central PMCID: PMC322383.
63. Defronzo RA. Glucose intolerance and aging: evidence for tissue insensitivity to insulin. Diabetes. 1979;28(12):1095-101. PubMed PMID: 510806.
64. Chavez AO, Coletta DK, Kamath S, Cromack DT, Monroy A, Folli F, et al. Retinol-binding protein 4 is associated with impaired glucose tolerance but not with whole body or hepatic insulin resistance in Mexican Americans. Am J Physiol Endocrinol Metab. 2009;296(4):E758-64. Epub 2009/02/05. doi: 90737.2008 [pii]
10.1152/ajpendo.90737.2008. PubMed PMID: 19190263.
65. Tantiwong P, Shanmugasundaram K, Monroy A, Ghosh S, Li M, DeFronzo RA, et al. NF-kappaB activity in muscle from obese and type 2 diabetic subjects under basal and exercise-stimulated conditions. Am J Physiol Endocrinol Metab. 2010;299(5):E794-801. Epub 2010/08/27. doi: ajpendo.00776.2009 [pii]
10.1152/ajpendo.00776.2009. PubMed PMID: 20739506.
66. Sriwijitkamol A, Christ-Roberts C, Berria R, Eagan P, Pratipanawatr T, DeFronzo RA, et al. Reduced skeletal muscle inhibitor of kappaB beta content is associated with insulin resistance in subjects with type 2 diabetes: reversal by exercise training. Diabetes. 2006;55(3):760-7. PubMed PMID: 16505240.
67. Liang H, Tantiwong P, Sriwijitkamol A, Shanmugasundaram K, Mohan S, Espinoza S, et al. Effect of a sustained reduction in plasma free fatty acid concentration on insulin signalling and inflammation in skeletal muscle from human subjects. J Physiol. 2013;591(Pt 11):2897-909. Epub 2013/03/27. doi: jphysiol.2012.247510 [pii]
10.1113/jphysiol.2012.247510. PubMed PMID: 23529132; PubMed Central PMCID: PMC3690693.
68. Varma V, Yao-Borengasser A, Rasouli N, Nolen GT, Phanavanh B, Starks T, et al. Muscle inflammatory response and insulin resistance: synergistic interaction between macrophages and fatty acids leads to impaired insulin action. Am J Physiol Endocrinol Metab. 2009;296(6):E1300-10. Epub 2009/04/02. doi: 10.1152/ajpendo.90885.2008
90885.2008 [pii]. PubMed PMID: 19336660; PubMed Central PMCID: PMC2692398.
69. Cinti S, Mitchell G, Barbatelli G, Murano I, Ceresi E, Faloia E, et al. Adipocyte death defines macrophage localization and function in adipose tissue of obese mice and humans. J Lipid Res. 2005;46(11):2347-55. Epub 2005/09/10. doi: M500294-JLR200 [pii]
10.1194/jlr.M500294-JLR200. PubMed PMID: 16150820.
70. Fink LN, Costford SR, Lee YS, Jensen TE, Bilan PJ, Oberbach A, et al. Pro-inflammatory macrophages increase in skeletal muscle of high fat-fed mice and correlate with metabolic risk markers in humans. Obesity (Silver Spring). 2013. Epub 2013/09/14. doi: 10.1002/oby.20615. PubMed PMID: 24030890.
71. Fink LN, Oberbach A, Costford SR, Chan KL, Sams A, Bluher M, et al. Expression of anti-inflammatory macrophage genes within skeletal muscle correlates with insulin sensitivity in human obesity and type 2 diabetes. Diabetologia. 2013;56(7):1623-8. Epub 2013/04/19. doi: 10.1007/s00125-013-2897-x. PubMed PMID: 23595247.
72. Martinez HG, Quinones MP, Jimenez F, Estrada CA, Clark K, Muscogiuri G, et al. Critical role of chemokine (C-C motif) receptor 2 (CCR2) in the KKAy + Apoe -/- mouse model of the metabolic syndrome. Diabetologia. 2011;54(10):2660-8. Epub 2011/07/23. doi: 10.1007/s00125-011-2248-8. PubMed PMID: 21779871.
73. Sun L, Yu Z, Ye X, Zou S, Li H, Yu D, et al. A marker of endotoxemia is associated with obesity and related metabolic disorders in apparently healthy Chinese. Diabetes Care. 2010;33(9):1925-32. Epub 2010/06/10. doi: dc10-0340 [pii]
10.2337/dc10-0340. PubMed PMID: 20530747; PubMed Central PMCID: PMC2928335.

ATTACHMENT F. INVESTIGATOR BROCHURE

ATTACHMENT G. GRADING SCALE FOR PHLEBITIS

ATTACHMENT H. SUMMARY OF PROTOCOL CHANGES

Date of Change	Version Number	Section Modified	Before Change	After Change
09-25-2014	1.1	Title Page	IND Number: Pending	IND Number: 123955-0001
09-25-2014	1.1	Table of Contents		Added Attachment G., page 33
09-25-2014	1.1	4.1, page 12	Typographical error: Stable body weight "±1%"	Corrected: Stable body weight "1.5%"
09-25-2014	1.1	5.8.2, page 15	After reconstitution and admixture, solutions of Eritoran may be stored for up to 24 hours at temperatures between 15-30°C (59 to 86 °F) or under refrigeration.	After reconstitution and admixture, solutions of Eritoran may be stored for up to 24 hours under refrigeration at temperatures between 2-8 °C (36.5 to 46.4°F)
09-25-2014	1.1	ATTACHMENTS		Added ATTACHMENT G. Summary of Protocol Changes
12-03-2014	1.2	Header	Incorrect version # on some pages	Version "1.2" all pages
12-03-2014	1.2	Title Page	NCT# Pending	NCT*02267317*
12-03-2014	1.2	Section 3.4 Potential Risks to Subject Safety, page 11		A CBC and lipid panel will be obtained on Day 3 of Infusion Visit to assess effects on blood cell count and lipid metabolism.
12-03-2014	1.2	6.3 Visit 3 – Infusion Protocol, page 17	Subjects will be given lunch at ~1PM, monitored for three hours, and discharged to home.	Subjects will be given lunch at ~1 PM, monitored for three hours including a CBC and lipid panel obtained to assess effects on blood cell count and lipid metabolism, then discharged to home
12-03-2014	1.2	4.1 Inclusion Criteria, item 6)	Premenopausal women in the follicular phase, non-lactating and with a negative pregnancy test.	Premenopausal women, non-lactating and with a negative pregnancy test.
12-03-2014	1.2	Title Page, Study Summary, Section 1.4, 5.1, and 5.7	Eritoran injection	Eritoran for injection (per Eisai request to be consistent)
12-03-2014	1.2	Table of Contents and ATTACHMENTS	ATTACHMENT E & G	Added placeholders for ATTACHMENTS A, B, C, D, and F for use when attachments are updated
12-03-2014	1.2	Section 5.1, Page 14	"Since these will be a double-blind studies, matching placebo vials will be used."	Deleted typo and entire sentence: "Since these will be a double-blind studies, matching placebo vials will be used."
12-03-2014	1.2	Title Page		Added IRB Tracking Number 20140498H
12-03-2014	1.2	Section 5.3, page 14	Study staff maintains source documents, data records and specimens in a de-identified method using only the unique Subject ID/Randomization number.	Study staff maintains specimens in a de-identified manner using only the unique Subject ID/Randomization number; source documents and data records are stored in electronic systems with restricted access and paper records in locked cabinets.
04-14-2015	1.3	Study Summary, Page 1 (5 of 43), Inclusion Criteria	Stable body weight (±1%) for ≥ 3 months; Age 18-60	Corrected typo: Stable body weight (±1.5%) for ≥ 3 months; Age 18-65
04-14-2015	1.3	Study Summary, Exclusion Criteria		1) Presence of glucose intolerance based on ADA criteria (lean/obese NGT)

				2) Hemoglobin A1c ≥ 8.5% (diabetic subjects only).
05-12-2015	1.3	3.1 Subjects, Page 11	~80 subjects will be consented assuming ~30% screen failure or withdrawals.	Corrected typo: ~90 subjects will be consented assuming ~30% screen failure or withdrawals.
04-14-2015 and 05-12-2015	1.3	3.4 Potential Risks to Subject Safety Page 12	A CBC and lipid panel will be obtained on Day 3 of Infusion Visit to assess effects on blood cell count and lipid metabolism. Catheters will be placed in an antecubital vein and in a hand vein for the euglycemic clamps.	The maximal amount of blood to be drawn during the entire study for any given subject will be approximately ≤ 534 mL. Hematology comprehensive metabolic panel with lipids and liver function tests will be obtained at the Infusion Visit to assess effects on blood cell count, liver function and lipid metabolism. IV lines. Intravenous catheters will be used for infusions and venipuncture.
04-14-2015	1.3	Section 4.1 Inclusion Criteria	One of these 3 groups: … ; or (iii) lean (BMI <26 kg/m2) normal glucose tolerant.	Added "Lean subjects have no family history of diabetes"
04-14- 2015	1.3	Section 4.1, Inclusion Criteria, Page 12 (16 of 43)	Stable body weight (±1%) for ≥3 months. Age 18-60	Corrected typo: Stable body weight (±1.5%) for ≥3 months. Age 18-65
04-14-2015	1.3	Section 4.2 Exclusion Criteria	1) Presence of glucose intolerance based on American Diabetes Association (ADA) criteria; 2) Non-steroidal anti-inflammatory drugs or systemic steroid use for more than 1 wk within 3 months 3) Current treatment with anticoagulants (warfarin), Aspirin (up to 325 mg) and clopidogrel will be permitted if these can be held for seven days prior to the biopsies	1) For non-diabetic subjects, the presence of glucose intolerance on OGTT based on American Diabetes Association (ADA) criteria will be exclusionary. 3) 2) Hemoglobin A1c ≥ 8.5% (diabetic subjects only) Removed abbreviation of "wk" to read more clearly. "Non-steroidal … 1 week within 3 months 4) Added "History of, coagulopathy will be excluded."
04-14-2015	1.3	Section 5.8.2, page 15	After reconstitution and admixture, solutions of Eritoran may be stored for up to 24 hours under refrigeration at temperatures between 2-8 °C (36.5 to 46.4°F)	Change storage instructions for reconstituted drug to allow time between admixture and infusion starting. "After reconstitution and admixture, storage of solutions of Eritoran will be limited to 4 hours at 15-30°C or up to 24 hours at 2-8°C." Do not freeze.
04-14-2015 and 05-12-2015	1.3	Section 5.2, Treatment Regimen, Page 15	Following a screening period, which can be up to one month, each subject will be admitted on 2 different occasions (separated by 3-4 weeks each) to receive…	Extend window to 3-6 weeks: Following a screening period, which can be up to one month, each subject will be admitted on 2 different occasions (separated by 3-6 weeks each) to receive…
04-14-2015 and 05-12-2015	1.3	Section 6.3, Page 17		Adjusted visit window and added blood draws for safety to Visit 3 and 5 per FDA recommendation. Visit 3: Within 3-30 days after Visit 2, subjects will return to the BRU

	1.3	Section 6.3 cont'd	Page18 Analyses: Blood/plasma: Insulin, FFA and tritiated glucose are assayed every 10 minutes during the clamp. Plasma cytokines …monocytes will be done on whole blood samples collected immediately before the first eritoran dose and just before the insulin clamp. To confirm the inhibitory effect of eritoran on TLR4, an ex vivo LPS stimulation test will be performed on whole blood samples (59).	for the first eritoran/vehicle infusion. ". Hematology, comprehensive metabolic panel, including liver function tests and lipid panel will be performed prior to the infusion at Visits 3 and 5 (on Days 1, 2 and 3). If LDL cholesterol increases more than 10% from Day 1 to Day 3 (on Visit 3 or 5), lipids will be re-assessed on the following visit. Changed time for admission from 9AM to 7AM. Changed start time for tritiated glucose to 5-6AM not 8AM. Blood/plasma: Insulin, FFA and tritiated glucose are assayed 18 times during the clamp. Plasma cytokines … monocytes will be done on whole blood samples collected immediately before the first eritoran dose (Day 1) and just before the insulin clamp (Day 4). To confirm the inhibitory effect of eritoran on TLR4, an ex vivo LPS stimulation test will be performed on whole blood samples (59).
04-14-2015 and 05-12-2015	1.3	Section 6.4, Visit 4	5-7 days after Visit 3 all subjects come to the BRU for inspection of the biopsy sites. Subjects will maintain an isocaloric diet and will refrain from regular exercise until the next infusion protocol.	5-7 days after Visit 3 …regular exercise until the next infusion protocol. Added: "If the lipid levels increased >10% during the Visit 3 hospital stay (from Day 1 to Day 3 measurements), we may repeat lipids (LDL cholesterol) at Visit 4."
04-14-2015 and 05-12-2015	1.3	Section 6.5 Visit 5	Approximately 3-4 weeks after Visit 4 all subjects come to the BRU to undergo the second infusion protocol (same as Visit 3).	Extend visit window: Approximately 3-6 weeks after Visit 4 all subjects come to the BRU to undergo the second infusion protocol (same as Visit 3).
04-14-2015 and 05-12-2015	1.3	Section 6.6 Visit 6	5-7 days after Visit 5 all subjects come to the BRU for inspection of the biopsy sites.	5-7 days after Visit 5 … the biopsy sites. Added blood draws at final follow up per FDA recommendation: ". If a 10% increase in lipid levels was observed during the Visit 7 hospital stay (from Day to Day 3), we may repeat the lipid levels (LDL cholesterol) on Visit 8. ."
04-14-2015	1.3	Section 8.6 Stopping Rules	In the unlikely event that a study-related death or SAE occurs, the decision to stop the trial, either temporarily or permanently, will be the responsibility of the	Added pre-specified clinical and laboratory parameters for stopping infusion or study participation (per FDA recommendation dated 12-23-2014). "Clinical or laboratory excursions that a subject may experience that may

			Designated Safety Officer in collaboration with the Sponsor Investigator.	warrant stopping study participation or drug administration, either temporarily or permanently, include but are not limited to: Clinical criteria: • Fever with a temperature >101°F • Allergic reaction to drug • Blood pressure >160/95 or <80/50 • Severe vomiting • Phlebitis grade 4-5 (see Attachment G.) Laboratory criteria: • Liver function test results >2x normal range • WBC ≥14,000 or ≤3,000 • HCT ≥55 or ≤33:" In the unlikely event…
04-14-2015	1.3	Section 9.2, Source Documents	Print all entries legibly in black ink.	Print all entries legibly in blue or black ink.
04-14-2015 and 05-12-2015	1.3	Section 12.3, Subject Stipends or Payment		Added an Unscheduled Visit Payment of up to $100 for unusual circumstance
04-14-2015	1.3	Table of Contents and ATTACHMENTS	Attachment C – Event Schedule	Changed Attachment C – Interval for infusion visits changed from 3-4 weeks to 3-6 weeks after previous visit. Added placeholder for ATTACHMENT G – Grading Scale for Phlebitis and moved Summary of Protocol Changes to Attachment H position
05-12-2015	1.3	Title page version number	Version 1.3 was modified twice, on 04-20-15 and 05-12-2015 (NM) without changing the version number	Should have been v 1.4 and dated 05-12-2015
06-15-2015	1.5	Title Page	Amended: v1.4_05-05-2105	Amended: v1.5_06-15-2015
06-15-2015	1.5	Header	Version 1.3	Version 1.5 (updated, was not changed with version 1.4)
06-15-2015	1.5	Footer	14-497H, Musi, Form BB, 05-07-2015, AMD	14-497H, Musi, Form BB, 06-15-2015, AMD (updated)
06-15-2015	1.5	Section 3.4		Added return to insert a line space between risk categories where none existed before
06-15-2015	1.5	Page 17	Extra white space before "For visit 3…" and after the figure "Schema of Visit 3.	Removed extra white space
06-15-2015	1.5	Section 6.3, Visit 3 – Infusion Protocol	"Subjects will be given lunch at 1PM…"	Remove phrase: "…including a CBC and lipid panel obtained to assess effects on blood cell count and lipid metabolism"…
06-15-2015	1.5	Section 8.2, Recording Adverse Events		Add sentence: "A summary of AEs tracked…"
06-15-2015	1.5	Section 8.3.1, Investigator reporting: notifying the		1) Add paragraph: "The Sponsor Investigator will comply…" 2) Change sentence regarding study-related UPIRSO: Replace "Any"

		study sponsor		with “A” and replace “and” with “involving” Add sentence: “A summary of non-serious AEs will be reported…”
06-15-2015	1.5	Section 8.4, Medical Monitoring		Add paragraph: “Twice a year the Sponsor Investigator will provide study safety information to the Designated Safety Officer…”
06-15-2015	1.5	Section 8.5.2-8.5.4	Medical Safety Monitor or Safety Officer	Replace with “Designated Safety Officer”
06-15-2015	1.5	Section 9.2, Source Documents	Print all entries with black ink	Change: Print all entries with “blue or black ink.”
08-05-2015	1.6	Title page, headers and footers	v1.5, <date>	Updated in all places to v1.6, 08-05-2015
08-05-2015	1.6	Study Summary	Study Duration… 4 -4[sic] months	Duration extended: 4-6 months
08-05-2015	1.6	Study Design	*Study Design.* The proposed study is a randomized, crossover, double blind, placebo-controlled study. Each subject will undergo 6 study visits over a period of 3 – 4 months.	Deleted sentence, kept subheading *Study Design*. Redundant statement of previous paragraph.
08-05-2015	1.6	Section 3.4, Potential Risk	Muscle Biopsy	Added: “There is the possibility that a future biopsy may not be done at the discretion of the PI in case the subject did not tolerate well a prior biopsy.”
08-05-2015	1.6	Section 6, Study Visits, pages 16-18	6.2…3-14 days 6.3, 6.5…3-30 days	Change: Extend visit window between V1-V2 to 3-45 days and V2 –V3 to 3-45 and 4-10 weeks between inpatient visits 3 and 5.
08-05-2015	1.6	Section 8.3.1	(formatting change)	¶3, page 21, word spacing and punctuation corrected
02-10-2016	2.0	Cover page and headers to update protocol version and IND version	v1.6 08-06-2015 (proposed change to inclusion/exclusion criteria)	Updated protocol versioning and serial IND numbers consistently: -Protocol v1.6 with 123955-0005 per FDA Annual Report 10-06-15 (advance in IND# due admin update of protocol to v1.6.1). -Protocol v1.6 with IND 123955-0006 to FDA on 11-12-15 extended drug expiry date (advance in IND due admin update of protocol to v1.6.2) -Protocol v2.0 will become IND 123955 -0007 per changes listed below
02-10-2016	2.0	Study Summary, Exclusion Criteria	v1.6 08-06-2015 - Current statement reads: “The only anti-diabetic agents allowed for T2DM subjects will be sulfonylureas because of their negligible anti-inflammatory properties.”	Page 1, Exclusion Criteria - Revised statement to read: “The only allowed anti-diabetic agents will be sulfonylureas and metformin.”
02-10-2016	2.0	Sections 4.2 and 5.6, Exclusion Criteria	v1.6 08-06-2015 – Current statement reads: “Subjects on sulfonylureas are eligible.”	Pages 13 and 16, Exclusion Criteria - revised statement to read: “Subjects on sulfonylureas and/or metformin are eligible.”
02-10-2016	2.0	Section 6.3 – Visit 3 Infusion Protocol	v1.6 08-06-2015	Page 18, adjusted spacing and lines to improve where page break occurs
02-10-2016	2.0	Section 7.1,	V1.6 08-06-2015 –	Page 19, revised statement to read:

		Statistical Plan	Current statement reads: "…enroll ~88 subjects."	"…enroll ~90 subjects" to be consistent throughout document and consent form.