Tauroursodeoxycholic Acid for Protease-inhibitor Associated Insulin Resistance
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Tauroursodeoxycholic acid for protease-inhibitor associated insulin resistance
PI: Reeds

A1. Abstract

The introduction of protease-inhibitor based antiretroviral therapy (PI-ART) has reduced the mortality associated with HIV infection (HIV+). Unfortunately, PI-ART use is a major risk factor for insulin resistance, an important risk factor for diabetes and coronary heart disease (CHD). Tauroursodeoxycholic acid (TUDCA), a naturally occurring bile salt, improves insulin sensitivity in insulin resistant people who do not have HIV. We have found that TUDCA markedly ameliorates ritonavir-induced insulin resistance in human myotubes and mice. The mechanism(s) responsible for these TUDCA-induced metabolic improvements are unclear, but could be related to: 1) TGR5 receptor activation, which upregulates cellular factors in muscle, including type 2 deiodinase (which increases intracellular triiodothryonine) and PGC-1 that increase mitochondrial biogenesis and fatty acid oxidation and/or 2) acting as chaperones that reduce endoplasmic reticulum (ER) stress by assisting protein folding. Initiation of ritonavir-boosted PI-ART worsens insulin sensitivity in HIV+ people and induces markers of ER stress in adipose tissue. We propose to perform a double-blind, randomized, controlled trial to determine if TUDCA improves insulin sensitivity in insulin-resistant, HIV+ people receiving PI-ART and to clarify the molecular mechanisms responsible for these improvements. We will randomly assign 48 insulin-resistant, HIV+ subjects to either placebo or TUDCA (1.75g/day x 30 days) and will measure multi-organ insulin sensitivity before and after the intervention by using a multistage hyperinsulinemic euglycemic clamp with infusion of stable isotope labeled tracers. To clarify the mechanisms responsible for the anticipated improvements in insulin sensitivity, we will examine the effect of TUDCA on TGR5 activation (type 2 deiodinase expression) in skeletal muscle biopsies and on ER stress by measuring Grp78 in adipose tissue biopsies taken before and after TUDCA administration. The results from this study will determine not only whether TUDCA may be effective for treatment of PI-associated insulin resistance but is also expected to identify new pathways by which TUDCA exerts insulin sensitizing effects. In addition to improving clinical care of people with HIV, the findings of this study may allow development of new drugs for treatment of type 2 diabetes.

Purpose of Study Protocol

The overall goals of this proposal are to evaluate whether TUDCA administration improves insulin sensitivity in HIV+ people receiving ritonavir-boosted, PI-ART, and to examine the potential cellular mechanisms through which TUDCA imparts its metabolic benefits. These findings will reveal novel molecular mechanisms by which TUDCA exposure improves insulin resistance in people living with HIV. Specifically, we will conduct a double-blind placebo-controlled trial to examine the effect of TUDCA (1.75g x 4 weeks) on multi-organ insulin sensitivity, muscle TGR5 signaling, and markers of adipocyte ER stress in 48 insulin-resistant, HIV+ men and women receiving ritonavir-containing PI-ART

B. Background

B1. Prior Literature

HIV infection is associated with increased risk of metabolic abnormalities. HIV+ individuals have a 2-4 fold greater risk for developing type 2 diabetes (T2DM) than the general population (1, 2) as well as dramatically increased rates of cardiovascular disease (CVD) (3, 4). Traditional risk factors including insulin resistance (IR)

are key predictors of CVD events in chronic HIV infection, and are especially important given the greater prevalence of T2DM in HIV-infected people (4-7). Protease-inhibitor based antiretroviral therapy (PI-ART) use, in particular use of ritonavir (RTV), is an established risk factor for development of IR and T2DM (1, 8, 9). Several PIs impair insulin-mediated stimulation of glucose uptake and suppression of glucose production and reduce insulin secretion (10-13). PIs can compete with glucose for binding to the facilitative glucose transporters expressed on skeletal myocytes (11). However, other intracellular molecular mechanisms are also likely at play. Nucleoside reverse transcriptase inhibitors are associated with reduced expression of genes critical in adipocyte development and mitochondrial biogenesis (PGC-1) (14-16). Indeed, we, and others, have reported a reduced capacity for fatty acid (FA) oxidation in HIV+ people with IR (17). Reduced lipid oxidation may promote tissue lipid accumulation and insulin resistance (17, 18). High concentrations of some PIs, including ritonavir, atazanavir and lopinavir, may also increase ER stress (19-25), which has been linked to development of insulin resistance (26-28), but this has yet to be demonstrated in vivo. Thus, the etiology of insulin resistance in treated HIV is likely multifactorial.

Drug and lifestyle intervention are less effective in cART-associated insulin resistance. Lifestyle change is recommended for obese, HIV+ insulin-resistant people (29, 30), however Engelson et al found that 7% weight loss did not improve IR in obese, HIV+ women (31). While exercise improves IR in HIV+ subjects, long-term changes in exercise habits are hard to maintain (32). Metformin and thiazolidinediones (TZDs) improve insulin sensitivity in HIV+ subjects (33, 34) but these drugs are associated with several side effects. Metformin often causes diarrhea and worsens subcutaneous fat loss, while TZDs may increase the risk of myocardial infarction, osteoporosis and worsen abdominal adiposity (33, 35-41). Furthermore, recent data indicates that HIV infected people, particularly those receiving PIs, have poorer glycemic responses to anti-diabetic drugs than HIV uninfected people (42). New, effective therapies are badly needed.

Bile acids (BAs) as an alternative therapy to increase insulin sensitivity. Studies conducted in cultured cells or in mouse models of obesity suggest that exogenous BAs improve insulin sensitivity (26-28). Members of our group performed a double-blind, randomized, placebo-controlled trial to examine the effects of the bile acid tauroursodeoxycholic acid (TUDCA) (donated by Bruschettini S.r.l.), which is approved for treatment of cholestatic liver disease in Europe, on insulin sensitivity in HIV uninfected, insulin-resistant subjects (43). Insulin sensitivity was measured by using clamp protocols identical to that contained in this proposal. *Treatment with 1.75 g/day of TUDCA for 30 days (a dose and duration identical to that proposed in this application) increased the hepatic insulin sensitivity index (Fig 1A) by 42% (0.86±0.17 vs 1.22±0.16 100/μmol/min x μU/ml, P<0.01) and insulin-sensitivity in skeletal muscle (Fig 1B) by 22% (Pre 48±5 vs Post 59±6 μmol/kgFFM/min, P<0.01 (Fig 1).* Western blot analyses performed on muscle biopsies from these subjects by Dr. Finck's lab, found that insulin receptor substrate-1 (IRS-1) phosphorylation and signaling (p-AKT) also improved (Fig 2), indicating that TUDCA administration ameliorates skeletal muscle insulin resistance at a molecular level. Furthermore, these and other studies conducted by Dr. Finck, found no indication that ER stress signaling was activated in skeletal muscle of insulin resistant subjects compared to insulin sensitive controls or that TUDCA reduced ER stress in

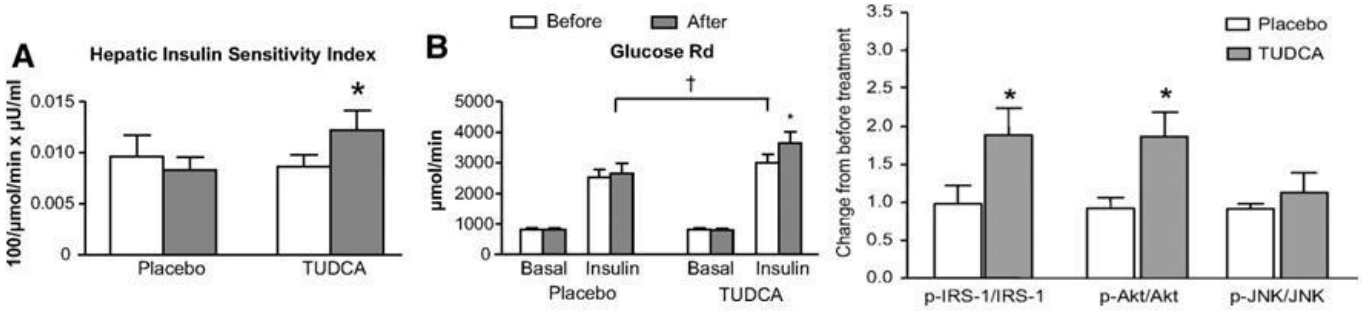

Fig 1 A and B Liver and muscle, insulin sensitivity before (☐) and after (■) 4 weeks of placebo or TUDCA treatment. *A*: Hepatic insulin sensitivity index. *B*: Glucose rate of disappearance (R_d). *Value significantly different from corresponding value before treatment, $P < 0.05$. †Main effect of insulin, $P < 0.0001$

Fig 2. Effect of placebo (☐) or TUDCA (■) treatment on muscle IRS^{Tyr}, Akt^{Ser473}, and $JNK^{Thr183/Tyr185}$ levels. Values are means ± SD and expressed relative to values before treatment,. *Value significantly different from corresponding placebo value, $P < 0.05$

these subjects (43). This raises the possibility that the effects of TUDCA are independent of effects on ER stress signaling.

Molecular actions of bile acids. The mechanism(s) whereby BAs mediate metabolic improvements are unclear but may include [1] activating cell surface receptors that activate intracellular signal transduction cascades (27, 28, 44-47), [2] acting as chaperones that reduce endoplasmic reticulum (ER) stress, and/or [3] serving as ligands for nuclear receptors that regulate gene transcription. This proposal is designed to address the two former mechanisms since, unlike other BAs, TUDCA has not been shown to have high affinity for any members of the nuclear receptor superfamily, and thus, this mechanism will not be evaluated.

TGR5, which is expressed in muscle, fat and liver, might mediate the metabolic effects of BAs. Recent work has suggested that BAs, including TUDCA, bind and activate the cell surface G-protein-coupled receptor TGR5. Several BAs, including TUDCA, exhibit high affinity for TGR5 and knockout of TGR5 abolishes the intracellular signaling response to BAs. In C57BL6 mice (*with normal TGR5 activity*), activation of TGR5 by BAs increases intracellular deiodinization of thyroid hormone by type 2 deiodinase (D2), converting tetra-iodothyronine (T4) to the more biologically active tri-iodothyronine (T3) which binds the iodothyronine receptor (TR) (44). This increases energy expenditure and fatty acid oxidation by stimulating expression of genes encoding fatty acid transport and oxidation (CPT-1) enzymes, and promoting mitochondrial biogenesis (PGC-1) (Fig 3). Studies in human skeletal muscle cells also showed that these effects were translatable to humans as well (44). *Our preliminary data show that TUDCA administration for 30 days increases expression of D2 in skeletal muscle in insulin-resistant, HIV uninfected subjects* (see *Innovation*). This proposal will evaluate the effects of TUDCA on markers of TGR5 action in skeletal muscle and adipose tissue of HIV+ subjects receiving PI-ART, clarifying whether this pathway may have therapeutic potential for other insulin resistant states. *The proposed*

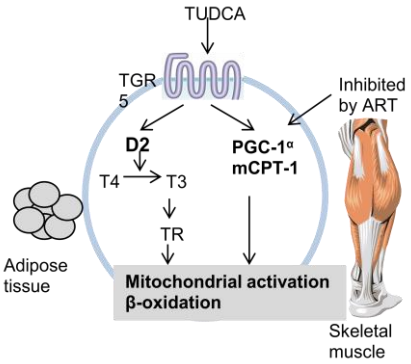

Fig 3. Mechanisms through which TUDCA may improve substrate metabolism.

studies will also use molecular genetic approaches to define whether TGR5 activation is required for the insulin sensitizing effects in skeletal myotubes exposed to PIs.

TUDCA may act as a chemical chaperone to assist with protein folding and reduce ER stress. During periods of cellular stress such as drug exposure to PIs, chronic viral infection (e.g. hepatitis C), or obesity, protein misfolding may occur in the ER and induce the unfolded protein response (UPR), which has been linked to the development of insulin resistance (27, 28, 48-50). The UPR activates three signaling cascades (Fig 4).

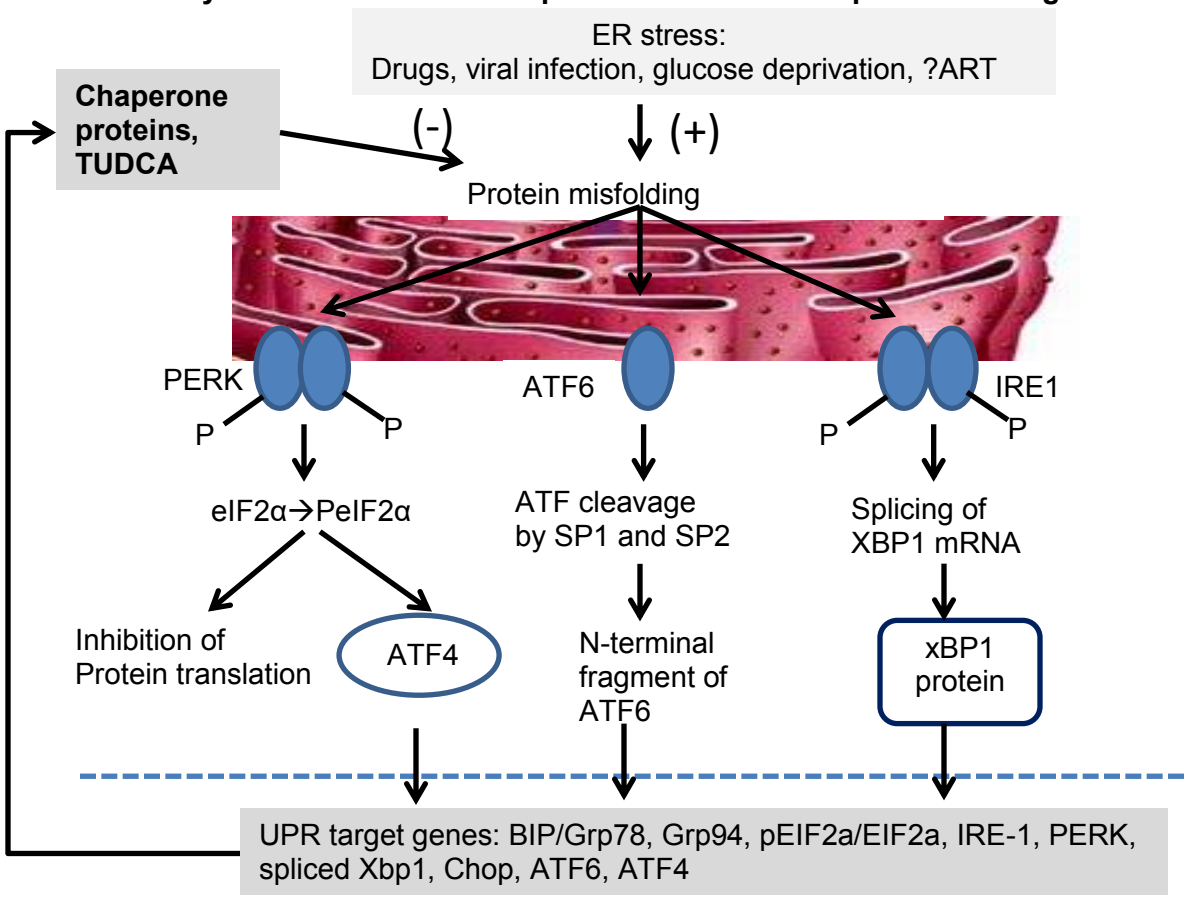

Fig 4. Schematic of ER stress and the UPR

1) *Release of the chaperone Bip (Grp78) into the ER* to assist protein folding and attenuate general protein synthesis by activation of PER- kinase (PERK), leading to phosphorylation of eukaryotic initiation factor 2α (eIF2α).

2) *Activation of inositol-requiring enzyme 1 (IRE-1)*, resulting in alternative splicing the XBP-1 transcript and the expression of XBP-1 protein, which in turn induces expression of a wide range of chaperone proteins which ameliorate protein misfolding. IRE-1 also activates JNK worsening insulin resistance (27, 51).

3) *Release of bound activating transcription factor-6 (ATF-6),* which induces XBP-1 expression and upregulates production of molecular chaperones. Through mechanisms that are still mostly unclear, TUDCA is known to reduce ER stress and is thought to act as a chemical chaperone to facilitate proper folding of misfolded proteins (28, 52, 53).

Besides its critical role in maintaining ER homeostasis, the UPR has an important impact on metabolic regulation and insulin signaling. Both defective and excessive activation of ER stress pathways have been linked to JNK activation, inflammation, and insulin resistance in several mouse models (17, 22, 23). Similarly, interventions that reduce ER stress, including TUDCA, improve insulin resistance in animal models (27, 28) and HIV uninfected

human subjects (43). Both HIV and PIs may activate the UPR and thereby contribute to metabolic dysfunction (19, 24, 54).

However, there is also evidence that the ameliorative effects of TUDCA on ER stress are not related to the insulin-sensitizing effects of this agent. For example, in Kars et al., we found that skeletal muscle glucose disposal and insulin signaling were improved in obese subjects, but found no evidence that ER stress was activated in skeletal muscle of these subjects or that TUDCA attenuated ER stress in this tissue (43). Our new preliminary data indicate that TUDCA improves insulin-stimulated glucose uptake in ritonavir-treated human skeletal myocytes, but we found no evidence that RTV caused ER stress in these cells. This could suggest other mechanisms whereby TUDCA elicits its effects. Therefore this proposal will clarify whether markers of ER stress are reduced in skeletal muscle and adipose tissue of HIV+ subjects after administration of TUDCA.

B2. Rationale for Study:

HIV+ people receiving PI-ART have an increased risk of DM and a blunted response to antidiabetic pharmacotherapy. Bile acids improve insulin sensitivity in animals and HIV seronegative humans, upregulate mitochondrial biogenesis, increase fatty acid oxidation, and may reduce ER stress, all of which are likely to have favorable metabolic effects. This proposal will use state of the art techniques to examine the hypothesis that the bile acid TUDCA will improve insulin-stimulated glucose disposal (i.e. insulin-sensitivity in skeletal muscle) in HIV+, insulin-resistant subjects receiving PI-ART. Further, we will clarify the cellular and molecular mechanisms by which TUDCA confers its benefits to test two hypotheses focused on the TGR5 and ER stress pathways. The mechanistic findings of this proposal will benefit insulin-resistant subjects irrespective of HIV serostatus.

Study Objectives

The overall goals of this proposal are to evaluate whether TUDCA administration improves insulin sensitivity in HIV+ people receiving ritonavir-boosted, PI-ART, and to examine the potential cellular mechanisms through which TUDCA imparts its metabolic benefits. These findings will reveal novel molecular mechanisms by which TUDCA exposure improves insulin resistance in people living with HIV. Specifically, we will conduct a double-blind placebo-controlled trial to examine the effect of TUDCA (1.75g x 4 weeks) on multi-organ insulin sensitivity, muscle TGR5 signaling, and markers of adipocyte ER stress in 48 insulin-resistant, HIV+ men and women receiving ritonavir-containing PI-ART

The following **Specific Aims** will be evaluated:

1. Determine the effect of TUDCA administration on multi-organ insulin sensitivity.
We hypothesize that TUDCA will improve *in vivo* insulin sensitivity more than placebo in skeletal muscle (glucose uptake) (primary endpoint), liver (suppression of glucose production), and adipose tissue (suppression of lipolysis), assessed *in vivo* by using a two-stage euglycemic-hyperinsulinemic clamp procedure with stable isotope tracer infusion and in the cellular components of the insulin signaling cascade (phosphorylation of IRS1 and Akt) and factors that inhibit insulin signaling (phosphorylation of JNK) in muscle and adipose tissue obtained by percutaneous biopsy .

2. Evaluate the effect of TUDCA on TGR5 activation and the role that this plays in the insulin sensitizing effects of TUDCA. We hypothesize that TUDCA treatment will increase skeletal muscle expression of D2 (primary endpoint), PGC-1α, and other markers of TGR5 activation. In addition, we hypothesize that in vitro knockdown of TGR5 will attenuate the beneficial effects of TUDCA

on PI-induced insulin resistance.

3. Determine the effect of TUDCA on endoplasmic reticulum stress in HIV+ men and women receiving PI-ART. We hypothesize that TUDCA will reduce adipose tissue markers of ER stress and inflammation (BIP/Grp78 (primary endpoint), Grp94, pEIF2a/EIF2a, IRE-1, PERK, spliced Xbp1, Chop, ATF6, ATF4, and phosphoJNK)

The results from this study will elucidate new intracellular pathways by which TUDCA exerts insulin sensitizing effects among HIV+ men and women receiving PI-ART, who represent a population at very high risk for diabetes and CHD.

D Investigational Agent

D1. Preclinical Data

TUDCA improves RTV-induced glucose intolerance in vivo. The primary endpoint of this application is to determine whether TUDCA will improve insulin-stimulated glucose disposal in HIV+ subjects treated with PI-ART. Ritonavir (RTV) boosted, PI-ART is the most commonly used PI therapy in our local HIV+ subjects (55) and is known to impair insulin sensitivity and risk of development of the metabolic syndrome (13, 56, 57). To begin to test the efficacy of TUDCA at ameliorating RTV-induced insulin resistance, wild-type, lean C57/BL6 mice were fasted overnight and then injected intraperitoneally TUDCA (500 mg/kg) or vehicle, one hour before receiving intraperitoneal RTV (1 mg/kg). This low dose of RTV dose, ~10% of that usually used in these experiments, was chosen because it is expected to achieve plasma concentrations similar to that seen with clinical use of ritonavir (10). An intraperitoneal glucose tolerance test (IPGTT) was performed 1 hour later (Fig 5A). Glucose AUC was greater in RTV (27477±1060 mg/dl x 120min), than the vehicle (22100±316, P=0.001), TUDCA (22500±928, P=0.001) and RTV+TUDCA (22902±372, P=0.001) groups. We also examined insulin signaling in fasted mice treated with TUDCA and RTV as in the IPGTT, but injected with insulin 10 min prior to sacrifice (n=2). In this pilot experiment, RTV treatment led to diminished Ser473 phosphorylation of Akt in gastrocnemius muscle, whereas TUDCA pretreatment reversed this effect of RTV (Fig 5B). These results indicate that TUDCA almost completely ameliorates RTV-induced glucose intolerance and impairments in insulin signaling in vivo.

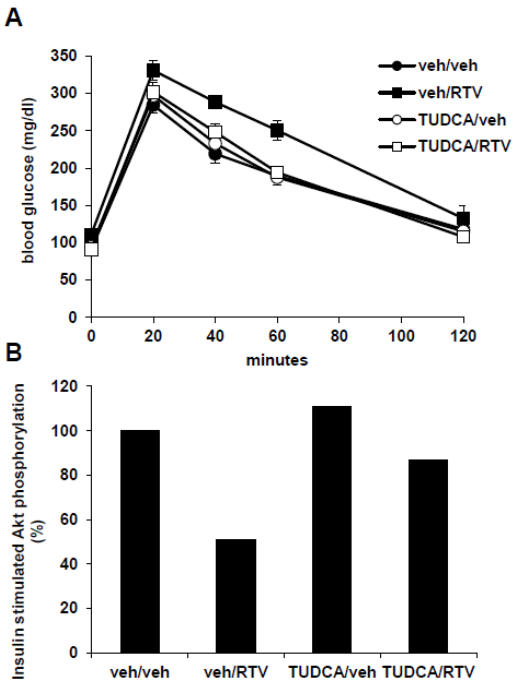

Fig 5 A. Plasma glucose concentration during IPGTT and [B] insulin-mediated phosphorylation of Akt Ser473 in skeletal muscle

TUDCA ameliorates RTV-induced insulin resistance in vitro. We examined whether TUDCA would reduce insulin resistance induced by RTV in human skeletal myotubes. Muscle satellite cells were isolated from vastus lateralis muscle biopsies obtained from HIV-seronegative subjects and were differentiated into myotubes for 10 days by Dr. Chen, as described previously (58, 59) On day 11, myotubes were then incubated with or without TUDCA (400 µg/ml) for 6 hours**.** RTV (10 µM) or vehicle (DMSO) were then added to the culture media and cells were incubated for ~8 hrs. ^{3}H-2-deoxy glucose

(2DG) was measured in the absence (basal) or presence of insulin (1 µg/ml). As shown in Fig 6, RTV reduced glucose uptake by ~50%, but this effect was partially reversed with pre-incubation with TUDCA.

In this pilot study, we specifically chose RTV concentrations were 2-3x that seen with boosting-doses to allow us to better delineate a potential maximum benefit of TUDCA on RTV-induced insulin resistance.

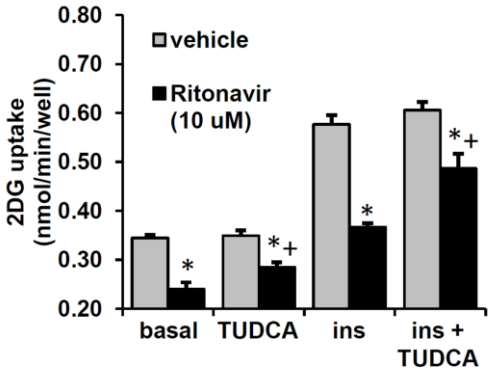

Fig 6. Effect of RTV±TUDCA on 2-Deoxyglucose uptake in human myotubes. Ins, insulin; T, TUDCA. * P<0.01 vs vehicle. + P<0.02 vs conditions without TUDCA.

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We also examined the effects of TUDCA on ER stress markers in human myocytes that had been treated with PIs (Fig 7). At the same doses and durations that impair basal and insulin stimulated glucose uptake (Fig 6), RTV did not activate EIF2a phosphorylation or Bip protein abundance (Fig 7). Other groups have shown that PIs induce ER stress in adipocytes and macrophages in vitro (19, 60). Indeed, lopinavir co-induced insulin resistance and ER stress in human adipocytes (19). On the other hand, the lack of effect of RTV on ER stress in skeletal myocytes is consistent with of the work of several groups showing that obesity-related insulin resistance is not associated with increased ER stress in skeletal muscle, but is associated with increased ER stress in liver and adipocytes (27, 43). These findings suggest that the insulin-sensitizing effects of TUDCA may be independent of its anti-ER stress mode of action.

Initiation of PI-ART induces ER stress in adipose tissue but not skeletal muscle. To expand upon this point, we determined whether initiation of PI-ART containing ritonavir led to indications of ER stress in humans. We performed western-blots for Grp78(Bip), EIF2α and phosphorylated EIF2α (pEIF2α) on skeletal muscle and adipose tissue biopsies from 7 HIV+ patients (5M, BMI 26±1kg/m^2), naïve to ART, with asymptomatic HIV infection, before, and 4 months after initiation of PI-ART (6ATV/RTV, 1 DRV/RTV, all receiving DF+FTC), which increased HOMA (1.7±0.1 vs 2.4±0.4, P=0.09) in these subjects. Although insulin resistance was worsened by PI initiation, skeletal muscle abundance of GRP78 and phosphorylated EIF2a was not affected by PI administration (Fig 8), which is consistent with our in vitro data.

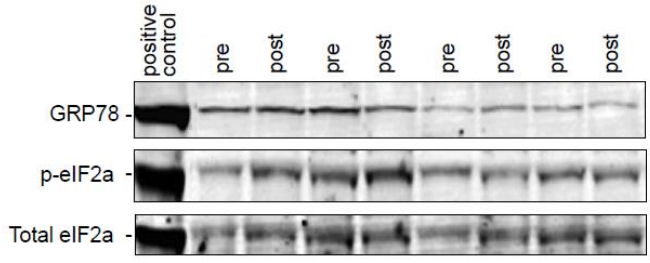

Fig 8. Skeletal muscle Grp78 abundance and peIF2α/peIF2α ratio before and after 4 months of PI-ART.

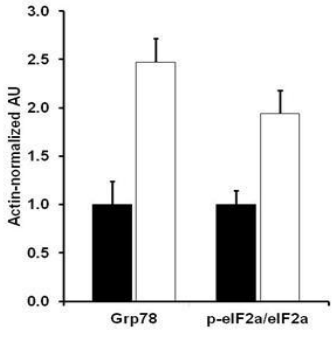

Fig 9. Relative change in adipose tissue Grp78 content and p-eIF2α: eIF2α ratio after 4 months of PI-ART .

* P<0.05,

+ P=0.07

In contrast, in adipose tissue, Grp78 abundance and the PeIF2α: eIF2α ratio increased after 4 months of PI-ART (Fig 9). These results suggest that PI-ART induces ER stress in adipocytes but not skeletal muscle in vivo in HIV+ humans. Recent work has suggested that dysfunctional adipose tissue, a term that encompasses a broad range of metabolic and inflammatory perturbations including ER stress, is involved in systemic insulin resistance by affecting circulating concentrations of metabolites, adipokines, and pro-inflammatory mediators. We have hypothesized that administration of TUDCA will reduce indications of ER stress in adipose tissue and will determine whether these changes are associated with improvements in multi-organ insulin sensitivity.

TUDCA increases expression of deiodinase 2 in skeletal muscle. Our alternative hypothesis is that TUDCA may be improving insulin sensitivity after RTV treatment via other mechanisms. Previous work has demonstrated that TUDCA is a potent activator of TGR5 signaling in human skeletal myocytes in vitro (44). To determine whether markers of TGR5 activation were also induced in human muscle at therapeutic doses, in collaboration with Dr. Abumrad's laboratory, we performed quantitative RT-PCR to quantify D2 mRNA expression in skeletal muscle biopsies from subjects treated with TUDCA (1.75/day, N=9) or placebo (N=9) for 30 days (Fig 10) (43). A strong trend towards increased expression of D2 was detected after 30 days of treatment with TUDCA (P=0.057), but was unchanged by placebo. These data provide evidence that TUDCA may impart its metabolic actions in part by increasing expression of D2 through TGR5 in skeletal muscle in humans in vivo. Our proposal will determine whether use of TUDCA causes similar changes in the setting of treated HIV, whether other TGR5 targets are activated by TUDCA in skeletal muscle in vivo (PGC1, CPT-1), and will use sophisticated in vitro approaches to define whether TGR5 is required for the beneficial effects of TUDCA on RTV-induced insulin resistance. These findings could potentially have a major impact on our understanding of how TUDCA improves insulin sensitivity in humans and assist with development of another class of antidiabetic agents.

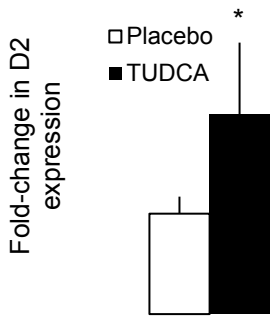

Fig 10. Fold change in D2 expression after placebo or TUDCA. *P=0.057 vs placebo

In summary, our preliminary data show:
1. RTV-induced glucose intolerance (and insulin resistance) is improved by treatment with TUDCA in mice.
2. RTV induces insulin resistance, but not ER stress, in human skeletal myocytes and RTV-induced insulin resistance is improved by treatment with TUDCA.
3. Markers of ER stress are increased in adipose tissue, but not skeletal muscle, biopsies of ART-naïve HIV+ subjects 4 months after initiation of PI-ART.
4. Treatment of insulin-resistant, HIV uninfected, human subjects with 1.75 g of TUDCA for 30 days induces markers of TGR5 activation in skeletal muscle.
The proposed mechanisms of insulin sensitization by TUDCA are not mutually exclusive and both pathways could be involved. TUDCA clearly elicits rapid ameliorative effects on ER stress in response to a variety of stimuli in liver and adipose tissue. However, prolonged (4 week) treatment with TUDCA

improves skeletal muscle insulin sensitivity in human obese subjects, who did not exhibit an activation of the ER stress response in that tissue (43). While the effect on skeletal muscle insulin sensitivity could be explained by peripheral effects on hepatic or adipose tissue metabolism or adipokine secretion, these effects could also be mediated by activation of TGR5 signaling in skeletal muscle. TUDCA may also have both acute and chronic actions or alternatively could be affecting ER stress pathways in a tissue-specific manner. By clarifying the mechanism(s) responsible for improvements in insulin sensitivity with TUDCA, this proposal will improve the quality of care for people living with HIV, but also benefit those with insulin resistance but without HIV.

D.2 Dose Rationale and Risk/Benefit

Natural bile salts consist of a family of amphipathic steroids produced by cholesterol metabolism in the liver. Bile salts are classified as primary (synthesized in the liver) or secondary (produced by bacterial transformation of bile salts within the gut). In man, primary bile salts consist of cholic acid (CA) and chenodeoxycholic acid (CDCA) and the secondary bile salts deoxycholic acid (DCA) and lithocholic acid (LCA). Ursodeoxycholic acid (UDCA) is an epimer of CDCA and is normally present in small amounts in human bile (0-5% of total pool) however taurine-conjugated UDCA (TUDCA) represents the physiologic form in which UDCA is secreted in bile. The taurine conjugation makes UDCA slightly more water soluble and makes precipitation of TUDCA within the gallbladder improbable due to the acidic conditions.

TUDCA has been licensed for the treatment of cholesterol gallstones and for therapy of chronic cholestatic liver disease in Europe since 1991 and is used at a dose of 10-20 mg/kg/day depending on the indication. Chronic toxicity has been studied in dogs for up to 26 weeks with treatment of up to 400-600 mg/kg/day (30 x that of the proposed dose) with no observed toxicity. Rats treated with 2-20 times the proposed dose showed no evidence of carcinogenic effects after 24 months of treatment. (Please see appendix).
Several recent studies indicate that 1.75g/day of TUDCA as proposed in this application is a safe and likely effective dose for insuiln resistance. In a recent study 10 obese, insulin-resistant adults received 1.75 g/day of TUDCA for 4 weeks with no adverse events. (43) A cohort of subjects with primary biliary cirrhosis were treated with 500-1500 mg/day of TUDCA for 6 months, with diarrhea reported as the only side-effect. Several human studies have shown that use of TUDCA is well tolerated in many chronically ill populations including amyloidosis(61), primary biliary cirrhosis (62-65) and liver transplant (66). Most experience with TUDCA has been in the use of this agent with liver disease, predominantly primary biliary cirrhosis (PBC). Crosignani et al treated 24 subjects with PBC with 500mg, 1000mg, or 1500mg of TUDCA per day. (63) All patients showed improvements in liver function tests with the greatest improvements seen in the 1000mg and 1500mg doses. The only reported side effect was diarrhea. The predominant side effects reported with TUDCA use in human studies have been diarrhea, headache, dyspepsia or abdominal pain. We exclude all patients with prior gallbladder disease or with diarrhea.

In summary, TUDCA has been tolerated at similar dose in humans with a variety of chronic medical conditions, with the major side-effects being mild diarrhea. The balance of the clinical information suggests that this drug is well tolerated and could have important health benefits for this study population. There are no known interactions between bile acids and antiretroviral drugs.

The dose proposed in this study is 1.75 g/day which will correspond to an average dose of 15-20 mg/kg/day. We wish to use this dose because this is a dose that has been shown to be effective in other human studies with few side effects. (43)
Based on available animal and human data, other than the potential for diarrhea, TUDCA appears to be very well tolerated even at doses 30-fold greater than that proposed. We therefore believe that this agent has shown very limited toxicity in humans but could confer significant cardiometabolic benefit.

E. Study Design

E.1

Specific Aim 1: Determine the effect of TUDCA on multi-organ insulin sensitivity.

Multi-organ insulin sensitivity will be assessed by performing euglycemic clamps, in conjunction with stable isotope labeled tracer infusion (section 4) and tissue biopsies, before and after placebo or TUDCA. We have predicted that treatment with TUDCA will improve skeletal muscle glucose disposal and insulin signaling in HIV+ subject treated with PI-ART.

1. Subjects. The study population will consist of two groups of patients (Fig 11):

Group 1 will consist of *24* subjects with HIV+ receiving RTV boosted PI-ART who have insulin resistance as evidenced by the presence of impaired fasting glucose (IFG), impaired glucose tolerance (IGT) or HOMA-IR>3.0 who will receive TUDCA 1.75g/day for 30 days.

Group 2 will consist of a total of *24* subjects with HIV+ receiving RTV boosted PI-ART who have IFG, IGT or HOMA>3.0 who will receive matching placebo for 30 days.

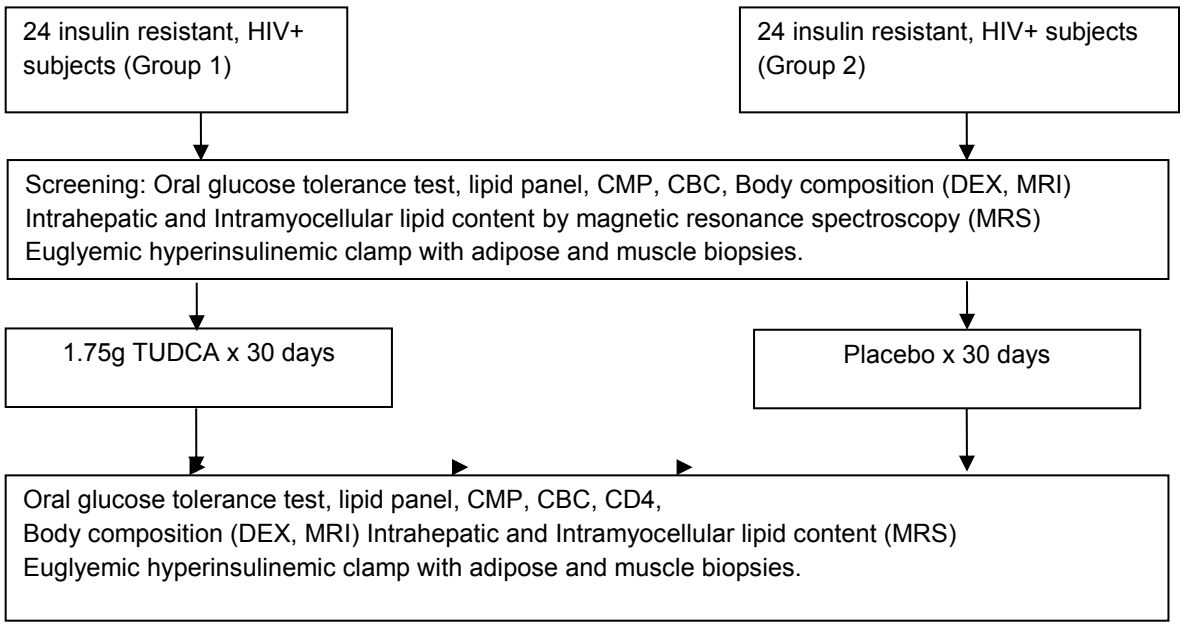

Fig 11. Experimental schema

2. Study Subjects:

Tauroursodeoxycholic acid for protease-inhibitor associated insulin resistance
PI: Reeds

E 2.a *The inclusion criteria are: 1) aged 18-70 yrs, 2) body-mass index of 20-40 kg/m^2, 3) have documented HIV infection with positive anti-HIV antibody and Western blot, 4) undetectable viral load for at least 6 months prior to enrollment, 5) hemoglobin > 10g/dl, 6) have abstained from agents that affect glucose and lipid metabolism for at least six months prior to enrollment (e.g. GH, glucocorticoids, omega-3-fatty acids, metformin), 7) have unchanged prescription medications for at least 3 months prior to enrollment, and 8) all subjects must be able to give informed consent.*

E 2.b *The exclusion criteria are: 1) history of eating disorder, 2) weight loss (>5% body weight in prior 6 months), 3) severe obesity (BMI>40kg/m^2), 4) significant GI disease (liver function tests >1.5x normal, persistent diarrhea, celiac disease, cirrhosis, hepatitis B or C), or gall bladder disorder that might alter glucose or bile-acid metabolism, 5) pregnant or nursing mothers, 6) history of diabetes mellitus, 7) history of serious cardiovascular disease; MI, angina pectoris, heart failure, coronary artery disease, stroke, uncontrolled hypertension, clinically significant valvular disease, prior endocarditis, 8) history of or active substance abuse (esp. history of alcoholism), 9) dementia or any condition that would prevent voluntary informed consent or compliance with research protocol, 10) debilitating myopathy or neuropathy, 11) coagulopathy, 12) new serious systemic infection during the 12 weeks prior to enrollment, 13) subjects with known allergy to bile salts, 14) subjects receiving drugs that interfere with bile acid absorption of metabolism including clofibrate, bile acid sequestrants (e.g. cholestyramine, welchol, ezetimibe) or 15) subjects whom the PI or investigative team do not believe are appropriate to include in the study*

Participants of childbearing potential and/or their partners must agree to use acceptable methods of birth control beginning at the pre-study visit and throughout the study. Acceptable methods of birth control include abstinence, depo-provera or norplant, or 2 of the following intrauterine device (IUD-with or without local hormone release), diaphragm, spermicides, cervical cap, contraceptive sponge, and /or condoms.

Study subjects will be recruited from the resources listed below.

Washington University ACTU: Dr. Presti provides clinical care at the ACTU. Since establishment in 1987, over 1700 enrolled in 263 clinical research projects at the ACTU. Since 2001, the ACTU has consistently enrolled 300-500 patients per year in HIV/AIDS clinical studies. The PI has worked with the ACTU for >10 years.

Vanderbilt Comprehensive Care Clinic and Vanderbilt Therapeutics Clinical Trial Unit (VTCRS): Drs Koethe and Hulgan provide care for HIV+ patients at the VUMS-ACTU. They have an excellent relationship with local ASOs. The PI has been working with Dr. Hulgan for the last 14 months to begin a collaborative relationship of screening patients at the VUMS-ACTU for this protocol. The research team at VUMS are famililar with all aspects of the study including adverse events (both actual and theoretical) and the current data. They are experts in the provision of clinical care of HIV+ patients and regularly perform metabolic studies within this population.

AIDS Service Organizations (ASO): The ACTU has excellent relationships with ASO's and acts as consultants to them on medical issues and provides speakers on a regular basis. The ACTU has a close collaboration with the St.Louis Effort for AIDS (EFA), the largest ASO in the St. Louis Metropolitan area and Blacks Assisting Blacks Against AIDS (BABAA) the major ASO working with African-American HIV-infected persons.

Community Advisory Board (CAB): The CAB consists of individuals from patient, health care and community constituencies serving the target population. Dr. Presti attends the monthly meetings, and presents current activities to seek input from CAB members about priorities for the community.

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St Louis HIV Primary Care Task Force: Dr. Presti is a member of the St Louis HIV Primary Care task Force; a group of physicians who meet regularly to discuss research studies. In combination they care for ~2000 patients with HIV in the St. Louis metropolitan area. Historically 30% of our subjects have been referred from this group.
Study to Understand the Natural History of HIV and AIDS in the Era of Effective Therapy (SUN trial): Dr. Presti is Director of the 'SUN' Study. 250 patients are enrolled in this five-year, CDC-funded, longitudinal cohort study to evaluate the metabolic effects of HAART. 53% percent are African American and 26% are women. Enrollment in SUN will facilitate co-enrollment in the current proposal.

3. Screening studies (Performed at WUSTL and VUMS-ACTU)

Medical examination. All subjects will be screened with a detailed history and physical examination, pregnancy test (*if female*) and CBC, lipid profile, liver panel and resting 12-lead electrocardiogram. CD4 T-cell count, and plasma viral load. *Oral glucose tolerance test.* After an overnight (12 h) fast, blood samples for plasma glucose, and insulin will be obtained immediately before and every 30 min for 2 h after ingesting a 75 g oral glucose load. The criteria of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus (67) will be used to determine diabetes..Other than these screening procedures, all other components of the study are performed at WUSTL.

Eligible subjects from VUMS-ACTU who are consented and are eligible will be discussed by the research team at VUMS-ACTU and the study PI, Dr. Reeds. If the subject meets eligibility criteria transport will be arranged by WUSTL for the patient to undergo body composition, assessment of intramyocellular and intrahepatic TG content and the inpatient isotope-infusion protocols at WUSTL. After completion of baseline inpatient testing they will be kept overnight and transported back to Nashville the following day. All eligible subjects will undergo baseline testing within 3 months of screening. Subjects will have their study medication dispensed at WUSTL and both WUSTL and the VUMS-ACTU will have a staff member (Rebecca Basham, RN, not involved in data analysis) unblinded to group assignment for safety. Subjects enrolled at VUMS-ACTU will return to VUMS-ACTU every ~7 days for a pill count, vital signs, and to assess for any medication intolerance or other AEs.

Body Composition and assessment of intramyocellular and intrahepatic TG content: Body composition will be measured within a week of the isotope infusion protocol. Total body fat and fat-free mass will be determined by dual energy x-ray absorptiometry (DXA) (Hologic QDR 4500, Waltham, MA) (68). Abdominal (subcutaneous and intra-abdominal) adipose tissue mass will be quantified by magnetic resonance imaging (69, 70). Intrahepatic TG content will be determined by proton MRS (3T whole-body system; Magneton Vision Scanner; Siemens, Erlanger, Germany) as previously described (71).

Cardiovascular structure (Performed at WUSTL)

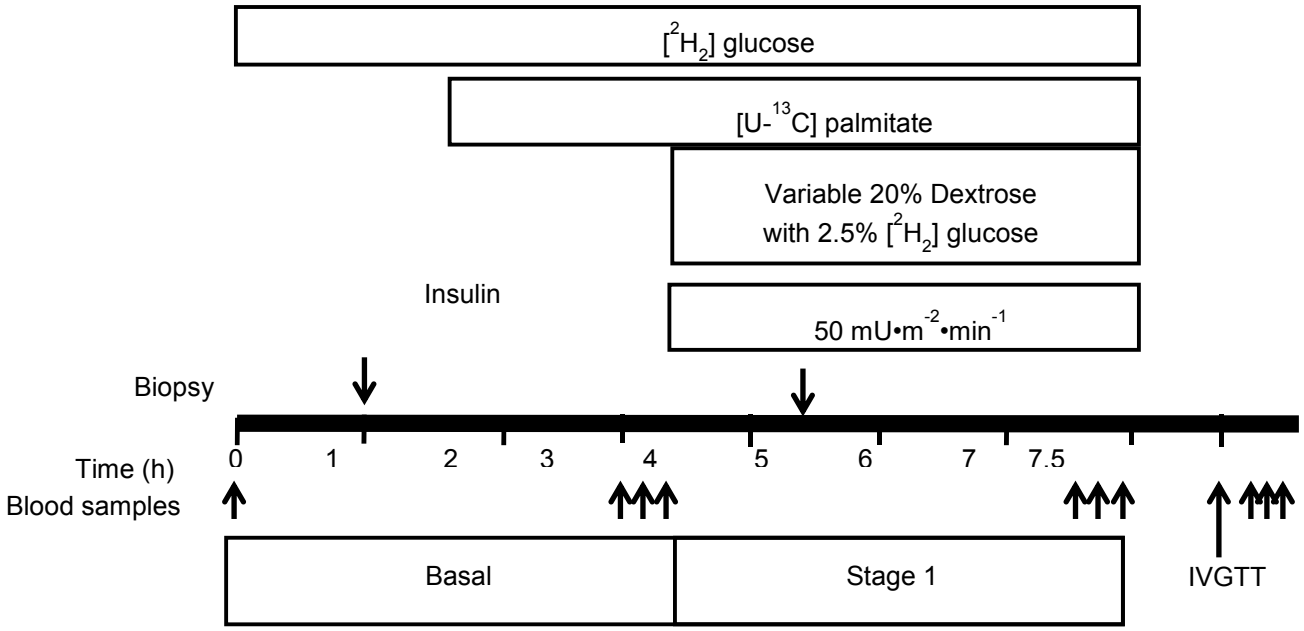
Cardiac echo and tissue Doppler imaging. Comprehensive resting echocardiography will be used to assess cardiac structure and function. This test is performed using a state-of-the-art ultrasound system (Vivid E9 Leadership or Vivid I Portable Cardiovascular Systems, GE Healthcare). Echocardiographic characterization will include assessments of left ventricular (LV) mass, LV size, LV systolic function, and LV diastolic function. LV mass will be determined by two methods: the 2D-derived LV mass indexed to body surface area and the 2D-guided M-mode-derived cubed method indexed to height$^{2.7}$ (LVM/Ht$^{2.7}$), which has been used extensively in clinical trials of hypertension (as recommended by the American Society of Echocardiography

guidelines for clinical trials) and has been shown to be highly reliable and a robust phenotype in overweight/obese populations [71-73]. The presence of LVH will be defined as an LVM/$Ht^{2.7}$ greater than two standard deviations above the mean (i.e. >47 g/$m^{2.7}$ for men, >44 g/$m^{2.7}$ for women) [74]. 2D-directed echocardiographic measurements include the LV end-diastolic and end-systolic volumes using the method of discs. The LV ejection fraction (LVEF) will be calculated using the modified Simpson's method (LVEF ≥55% considered normal). LVDD will be characterized as follows: 1) PWD-derived transmitral indices recorded from the four-chamber view at the mitral valve leaflet tips, including the early diastolic (E-wave) and atrial (A-wave) velocities, E/A velocity ratio, E-wave deceleration time (DT), and the isovolumic relaxation time (IVRT) [75]; 2) Tissue Doppler Imaging (TDI)-derived early diastolic myocardial velocity (E') obtained at the septal and lateral mitral annulus from the apical four-chamber view and the averaged value [75-77]. TDI-derived indices of LV diastolic relaxation (i.e., E' velocity) are relatively load-independent and inversely correlate with the degree of interstitial fibrosis [78-81]. Consistent with the recommendations of the American Society of Echocardiography for clinical studies of patients with hypertension, TDI-derived early diastolic mitral annular velocity (E') will be used as the primary assessment of diastolic function [73]. When dichotomization of LVDD is necessary for analysis, an E' <9.0 cm/s will be considered evidence of impaired LV relaxation. Measurements will be reported as the average of values from 3 consecutive cardiac cycles.

5. Isotope-Infusion Protocols.

Pre-study dietary intake and physical activity. All subjects will be instructed to abstain from any exercise and to ingest a standard weight-maintaining diet for three days before the isotope infusion protocol. Our study dietician will give dietary instructions individually to each subject. Each subject will keep a complete 3-day food log to judge compliance. The diet will contain at least 300 g of carbohydrate/day and the relative contributions from protein, carbohydrate, and fat will be approximately 15%, 55%, and 35% of calories, respectively. On the third day of the diet, subjects will be admitted to the IRU at 1600 h and their 3-day diet records will be reviewed. If their diet is inappropriate (e.g. inadequate in carbohydrate or energy intake) the study will be rescheduled. The study's dietary counselor will provide counseling by phone to participants recruited from the Vanderbilt site.

Hyperinsulinemic euglycemic clamp. Subjects will be admitted to the Clinical Research Unit (CRU) for isotope infusion studies to assess insulin sensitivity in liver, adipose tissue and skeletal muscle (Fig 12). At 1800 h on the day of admission, subjects will be given a standard meal (containing 15 kcal/kg FFM), which will be consumed by 1900 h. Subjects will then receive a can of Boost™. After this meal, subjects will fast until completion of the isotope infusion studies the following day. At 0500h, a catheter will be inserted into the antecubital vein of the arm to infuse isotope tracers and another catheter will be placed in the contralateral dorsal hand vein. The hand will then be placed in a thermostatically controlled box heated to 55C to obtain arterialized blood samples. If a hand vein cannot be cannulated, a radial arterial line will be placed. We have found that a radial artery catheter is well tolerated by study subjects, and ensures rapid and secure blood sampling. We now have inserted hundreds of catheters without any complications.



At 0600 h a hyperinsulinemic-euglycemic clamp procedure with isotope infusion will be initiated to determine hepatic (glucose production), skeletal muscle (glucose disposal) and adipose tissue (lipolysis) insulin sensitivity. After baseline blood samples are obtained, [6,6-$^{2}H_2$]glucose (22 μmol/kg prime and 0.22 μmol·kg^{-1}·min^{-1} constant infusion) will be infused. After 90 min (0730 h), a constant infusion of [U-^{13}C]palmitate (6 nmol·kg FFM^{-1}·min^{-1}) will be started to determine the rate of appearance (Ra) of plasma palmitate . At, 0930 h, after the basal period is completed, a two-stage euglycemic, hyperinsulinemic clamp will be initiated and continued for 6.5 h. Euglycemia will be achieved by a variable rate infusion of

Fig 12. Schematic diagram of isotope infusion protocol. IVGTT (intravenous glucose tolerance test) blood draws every 2 minutes for 8 minutes.

20% dextrose enriched to approximately 2.5% with [6,6-$^{2}H_2$]glucose to minimize changes in glucose isotopic enrichment. Adding tracer to the dextrose infusion has been shown to provide more accurate measures of glucose Ra (72), by minimizing changes in plasma glucose enrichment. During stage 1 of the clamp (3.5 h to 6.5 h of isotope infusion), insulin will be infused at a rate of 7 mU·m^{2}·min^{-1} for 3 h (initiated with a two-step priming dose of 30 mU·m^{2}·min^{-1} for 5 min followed by 15 mU·m^{2}·min^{-1} for 5 min), which will achieve plasma insulin concentrations of ~25 μU/ml. During stage 2 of the clamp (6.5 h to 10 h of the isotope infusion), insulin will be infused at a rate of 50 mU·m^{2}·min^{-1} for 3.5 h (initiated with a two-step priming dose of 200 mU·m^{2}·min^{-1} for 5 min followed by 100 mU·m^{2}·min^{-1} for 5 min), which will achieve plasma insulin concentrations of approximately 80 μU/ml. These plasma insulin concentrations provide an optimal range for evaluating insulin's effect on hepatic glucose production and lipolysis (low-dose insulin infusion) and glucose uptake by skeletal muscle (high-dose insulin infusion). The infusion of [$^{2}H_2$]glucose and [U-^{13}C]palmitate will be decreased by 50% of basal during stage 1 and by 75% during stage 2 because of the expected decreases in hepatic glucose production and lipolytic rate. Plasma samples will be taken before beginning the infusion to obtain baseline measurements of substrate enrichment. Plasma samples will be taken every 10 min during the last 30 min of the basal period and

each stage of the insulin clamp to determine glucose and FFA concentrations and kinetics, and insulin concentrations. Plasma samples will be obtained every 10 min at the end of the basal period and throughout the entire clamp to monitor plasma glucose concentration.

At the completion of the clamp, the insulin will be turned off . Plasma glucose will be checked every 10 minutes and the rate of 20% dextrose infusion will be reduced by 50% if BG >100mg/ml. After the 20% dextrose is off PG is checked every 10 min until BG returns to baseline. A bolus of 0.3g/kg of dextrose will then be given intravenously over one minutes. Bloo samples will be taken every 2 minutes for 8 minutes total to measure plasma insulin and c-peptide concentrations. In the event of hypoglycemia during the clamp (BG<60mg/dl) the insulin will be turned off, 20 grams of glucose will be given intravenously and the D20 rate will be titrated to achieve BG>100mg/dl. The study will then be aborted and the subject will be fed and observed as described above.

Oxygen consumption and carbon dioxide production will be measured by indirect calorimetry at 0900, 1200 and at 1600 h, for 30 minutes by using a metabolic cart and a ventilated hood system (Parvomedics TrueOne2400, Parvomedics, Sandy, UT). These data will be used to examine energy expenditure and substrate oxidation.

Tissue Biopsy: Tissue will be obtained from subcutaneous abdominal adipose tissue and the *vastus lateralis* muscle 60 min after starting the glucose tracer infusion (basal stage of the clamp procedure). Muscle and fat biopsies will also be obtained 60 min after starting stage 2 of the clamp procedure to determine the effect of insulin infusion on insulin signaling and adipocyte markers. The biopsy sites will be cleaned and draped in a sterile manner. After anesthetizing the area with lidocaine, adipose tissue will obtained aspirated through a liposuction cannula, ~200 mg of muscle will be obtained by using a 6 mm Bergstrom needle inserted through a small (0.5 cm) skin incision. All tissue samples will be immediately rinsed with ice-cold saline. Adipose tissue samples will be divided into 2 aliquots: 1) immediately frozen in liquid nitrogen for subsequent determination of cell factors and quantitative PCR 2)) placed in formalin for subsequent microscopy and immunohistochemistry analyses The PI has performed several hundred of these procedures and they are well tolerated by subjects.

Analysis of Samples. All analytical procedures required for this proposal are routinely performed in Dr. Klein's, Dr. Finck's, and Dr. Abumrad's laboratories, the CRU Core Laboratory, the Nutrition and Obesity Research Core (NORC) and the Adipocyte Core Biology Laboratories. Screening laboratory specimens obtained from participants who complete the screening visit at the Vanderbilt site will be processed by LabCorp using commercially available assays (Birmingham, AL)".

Plasma glucose and palmitate, kinetics*:* The resources of the NORC will be used for sample analysis. The lab has streamlined methods to process plasma to recover these substrates and derivatize them for GC/MS analyses (69, 70, 73-75). Steele's steady state equation(76) will be used to calculate substrate kinetics.

Molecular correlates of insulin sensitivity: We have hypothesized that TUDCA administration will improve insulin sensitivity in HIV+ subjects receiving ART. To evaluate molecular indicators of this, the phosphorylation status of several proteins that are downstream of the insulin receptor signaling cascade will be assessed, including phosphoserine473 and total Akt/protein kinase B (PKB) and total, phosphotyrosine, and phosphoserine IRS-1 by immunoblotting. These endpoints will be assessed during baseline conditions and during stage 2 of the clamp in skeletal muscle biopsies and quantified as

described just above. *Briefly, total cellular proteins will be extracted by mechanical disruption using a Tissue-Lyzer in lysis buffer containing protease and phosphatase inhibitors. Protein concentration will be determined and proteins electrophoresed under denaturing conditions.* Western blot results will be visualized and band intensity quantified using the Licor Odyssey system provided by the NORC. The intensity of the phosphorylated forms of the proteins are corrected for total content of that protein and normalized to the baseline value. Dr. Finck's laboratory performs these analyses routinely (77-80) including in the prior TUDCA study (43).

Measurement of serum bile acid concentration: Plasma bile acids will be measured using high-performance liquid chromatography tandem mass spectrometry (81)

Other assays: Plasma insulin, CRP, adiponectin, leptin, TNF α and IL-6 will be measured by the CRU Core laboratory using commercially available assays (Linco, St Charles, MO).

Specific Aim 2: Determine the effect of TUDCA on markers of TGR5 activation.

A major strength of this proposal is the systematic and rigorous examination of the molecular mechanisms that are likely to be responsible for the metabolic effects of TUDCA. Our leading hypothesis is that TUDCA enhances insulin sensitivity in skeletal muscle by activating the G-protein coupled cell surface receptor TGR5. This hypothesis will be tested by examining the mRNA expression and protein abundance of TGR5 targets in subjects before and after treatment with TUDCA (or placebo). We will then determine the requirement for TGR5 signaling for the response to TUDCA by using siRNA-based TGR5 silencing.

In vivo studies: We will evaluate markers of TGR5 activation (PGC-1α, several genes encoding fatty acid oxidation enzymes, enzymes involved in electron transport, and type 2 deiodinase) in adipose and skeletal muscle tissue samples taken before and after administration of TUDCA at the levels of protein abundance and mRNA expression. Protein will be isolated in the presence of protease and phosphatase inhibitors as described in Aim 1. The protein content and/or phosphorylation status will be performed by using western blotting. The antibodies above are all commercially available and have been used previously by Dr. Finck's lab (43, 74, 77-80, 82-84). Total RNA will be isolated from tissue biopsies using RNAzol Bee as we have described numerous times previously (43, 74, 77-80, 82-84). Quantitative RT-PCR will be performed by using reagents from Applied Biosystems and the ABI 7500 provided by the NORC Adipocyte Biology Core. Muscle mitochondrial DNA content will also be determined by back-extracting total DNA during RNA extraction and measuring the DNA for the mitochondrial gene, Cox1, and normalizing to nuclear DNA content by measuring RPLP0 DNA, which is encoded in the nucleus. Primer sets have been validated to specifically detect mRNAs of interest. *We predict that TUDCA treatment will lead to increased expression and protein abundance of markers of TGR5 activation in human skeletal muscle.*

In vitro studies: If our preliminary data hold true and the analyses described just above show that TUDCA administration activates multiple markers of TGR5 activation, cell biological approaches will be used to determine whether TGR5 is required for the ameliorative effects of TUDCA on insulin signaling and glucose uptake. Human skeletal satellite cells will be isolated and differentiated into myocytes in culture as described in the preliminary data. TGR5, which is well expressed in skeletal muscle cells (44), will then be knocked down by using siRNAs purchased from Sigma. We do not consider this to be a significant

technical hurdle as this technique is used frequently and successfully by Dr. Finck and Dr. Chen (78, 82-86) and Sigma will continue to provide oligonucleotide siRNA candidates until successful knockdown is achieved. After identifying efficient siRNAs to knockdown TGR5, differentiated myocytes will be treated with siRNAs to TGR5 (or scrambled siRNA control) for 72 h, myocytes will be treated with RTV (or vehicle) with or without pre-treatment with TUDCA as described in preliminary studies. Endpoints will include assessment of protein and mRNA markers of TGR5 activation and ER stress. Subsequent studies will include insulin stimulation to assess phosphorylation of insulin signaling cascade proteins and uptake of 2-deoxyglucose (as described in preliminary studies). *We predict that knockdown of TGR5 will abolish the activation of D2 and other proteins under the control of this signaling pathway. In addition, if TGR5 activation is important to the insulin-sensitizing effects of TUDCA, loss of TGR5 may also attenuate the ability of TUDCA to increase insulin sensitivity in these skeletal myotubes.*

Specific Aim 3 Determine the effect of TUDCA on ER stress.

Alternatively or in addition, we have hypothesized that TUDCA may improve insulin signaling by reducing ER stress, possibly in a cell type-specific manner. To test this hypothesis, we will evaluate markers of ER stress in tissue biopsy samples taken before and after administration of TUDCA. We will also evaluate the direct effects of TUDCA and RTV on these endpoints in adipocytes in culture to begin to dissect the molecular mechanisms involved.

The proposed methods will examine all three major pathways of the UPR; specifically we will assess 1) activation of PERK by measuring protein abundance of PERK, pEIF2a, EIF2a, and ATF4, 2) ATF6 activation by measuring the protein abundance of ATF6 and 3) Xbp1 splicing by measuring spliced Xbp1 content. We will also assess downstream targets of these pathways by measuring protein content of BIP/Grp78 and activation of the JNK pro-inflammatory pathway by measuring phosphoJNK.

In vivo studies: ER stress markers will be quantified in adipose tissue and skeletal muscle of study subjects. Protein and RNA will be isolated as described above and markers evaluated by using western blotting and qRT-PCR. We will assess various markers by protein abundance or phosphorylation status (BIP/Grp78, Grp94, pEIF2a/EIF2a, IRE-1, PERK, spliced Xbp1, Chop, ATF6, ATF4, and phosphoJNK). The splicing of Xbp1, which is increased by ER stress will be assessed by using SYBR chemistry quantitative reverse transcriptase-PCR (Applied Biosystems). Relative abundance calculated using the delta-Ct method ($2^{-\Delta Ct}$) with a housekeeping gene (RPLP0). Total RNA will be extracted from frozen muscle and adipose biopsy samples as described (87). Adipose tissue samples taken before and after the intervention will be analyzed for macrophage and inflammatory gene expression (IL-6 and TNF-α expression, F4/80, CD68, EMR1,CD11b, MIP-1a, CCL3-R) to determine if administration of TUDCA affects markers of adipose tissue inflammation. We predict that treatment with TUDCA will reduce signs of ER stress in adipose tissue, but not skeletal muscle, of study subjects treated with PI-ART.

In vitro studies: If, as predicted, TUDCA reduces ER stress in adipose tissue above, the effects could be direct effects of TUDCA as a chemical chaperone in adipocytes or could be secondary to systemic improvements in insulin sensitivity and metabolism. To address this issue, the human adipocyte cell line (Lisa-2; provided by the NORC ABC) will be cultured and differentiated in vitro. Adipocytes will then be treated with RTV and/or TUDCA as described for the human skeletal myocytes in prelim studies. Endpoints will include markers of ER stress, insulin signaling, and 2-DG uptake. Based on preliminary studies and published work showing that PI-ART activates ER stress in adipocytes (19, 88, 89) we predict

that treatment of adipocytes with RTV will activate the ER stress response and impair insulin signaling and glucose uptake and that TUDCA will attenuate or reverse these effects of RTV.

Finally, it is also possible that the ability of TUDCA to ameliorate ER stress could be blocked by loss of TGR5 signaling. Though this would seem to be a more unlikely possibility, the mechanism whereby TUDCA ameliorates ER stress is not known, and could involve TGR5 signaling. To test this ideas, TGR5 will be knocked down by using siRNA (as described for myocytes) and the ability of TUDCA to ameliorate ER stress and improve insulin responsiveness in adipocytes will be examined. These mechanistic studies would provide a significant conceptual advance for the field by establishing the mechanism whereby these effects occur.

E 2.C

Randomization. Participants will be randomized to TUDCA or placebo. A computer-based randomization scheme will be established, as used in our prior research protocols (90) and *group assignment will be stratified by race and gender. The study statistician, Dr. Schechtman, who will be unblinded to group assignment, will provide ongoing oversight to ensure groups are well matched on key characteristics (age, BMI, race, gender).* Capsules containing TUDCA and matching placebo will be made by our research pharmacy, as has been performed in our other placebo-controlled studies (90). The PI, subjects and research technicians will be blinded with respect to group assignment. The coordinator will also be unblinded to group assignment. In the event of an adverse event the coordinator will inform the team and subject of group assignment. Melisa Moore RN and Rebecca Basham RN will be unblinded to ensure that group assignment is known in the case a subject requires urgent medical care.

Sample Size and statistical power: All power computations are based on two-sided tests at the 0.05 level of significance for the *primary outcomes* of change of 1) insulin-stimulated glucose disposal (**SA#1**), 2) type 2 deiodinase expression in skeletal muscle (**SA#2**) and 3) Grp78 expression in adipose tissue (**SA#3**) after the intervention.

Significance of primary outcomes: Change in insulin-stimulated glucose disappearance is clinically relevant as a predictor of diabetes risk and is highly reproducible in our hands, improving statistical power (91). D2 and Grp78 expression are robust markers of TGR5 activation and ER stress, respectively, and will therefore clarify the mechanisms through which TUDCA may act to improve insulin sensitivity.

Estimates of change in the primary outcomes: The estimates for the anticipated changes in the primary endpoints of glucose disposal and D2 expression are based on the prior study conducted in obese, insulin resistant subjects. In this study, glucose rate of disappearance improved by 30±23% and D2 expression in muscle increased 2.4±2.5 fold (Fig 10) after the TUDCA intervention (43). The estimate for the change in Grp78 expression following initiation of TUDCA is based on improvements seen in animal studies with use of TUDCA (52, 53) and in humans after undergoing gastric bypass (92). Based on these findings we estimate a 50±25% change in Grp78 in the TUDCA group with no change in the placebo group.

G

G1. Statistical analysis: Preliminary analyses will include analysis of variance and chi square tests that focus on ensuring that the groups are similar at baseline. The primary analytic approach will be analysis of covariance in which the dependent variable will be the post intervention value of the outcome measure

and the independent variables will be the study group and the baseline value of the outcome. This will allow us to test hypotheses about the equality of post intervention values after adjusting for the baseline value. In these analyses, we will be specifically interested in the statistical contrasts that compare the groups with and without TUDCA. The appropriateness of all analysis of variance and covariance models will be evaluated using regression residuals. If normality or homoscedasticity requirements are violated, data transformations will be explored and, if appropriate transformations cannot be found, analyses may be done semi-parametrically using the ranks of the data.

G2. Power Estimates for each primary endpoint: We have conservatively estimated the improvements in our primary endpoints. *We plan to include 24 subjects in each group and conservatively estimate a 33% drop-out rate.* These drop-out rates are greater than those seen in our prior studies in HIV+ people using longer duration interventions that have high drop-out rates such as exercise (10% drop-out) or yoga (15% drop-out) (32, 93).

The above data have the following implications with respect to statistical power (see Table). The anticipated treatment induced improvement in the glucose disappearance rate of 30 $\pm$ 23% implies that a sample size of only 16 yields a power of 0.95 **(SA#1).** The expectation that TUDCA will increase D2 expression in muscle by 2.4 $\pm$ 2.5 yields a power of 0.85 with 16 subjects per group **(SA#2)**. Finally the expected change of 50 $\pm$ 25% in Grp78 in the TUDCA group implies that only 7 subjects per group are needed to achieve a power of 0.93 **(SA#3)**. In summary, our sample size selection implies that the power associated with the glucose disappearance rate (SA#1) and Grp78 (SA#3) will exceed 0.9 while the power associated with D2 expression will be 0.85.

G3. Statistical power estimates

Variable	HIV+ Placebo	HIV+ TUDCA	Power for detecting differences between groups after intervention (30% dropout)
Improvement in insulin-stimulated glucose disposal (%)	0±20	30±23	0.95 (if N=16 per group)
Fold-change in D2 expression after intervention	0±1.8	2.4±2.5	0.85 (if N=16 per group)
Fold-change in Grp 78 expression after intervention	0±0.25	-0.50±0.25	0.97 (if N=16 per group)

Study Timetable:

	Subjects
Year 1	8
Year 2	12
Year 3	12
Year 4	12
Year 5	4
Total studies	**48**

H. Protection of human subjects.

H1. Risk to subjects. Blood and tissue samples will be collected from all subjects. The specimens will be obtained solely for research purposes. Study samples and data sheets will be coded with an identification number for each subject. All of the data from individual subjects will be maintained confidentially and their names and identities will not be disclosed in any published document. The PI, coordinator and Dr. Presti are covered by a certificate of confidentially carried by the ACTU.

Potential risks of the study include: 1) the risks of catheter insertion and blood drawing include discomfort, bleeding or infection at the site of insertion; 2) the infusion of hormones may cause changes in blood glucose and may cause nausea, however, blood glucose is monitored throughout the study; 3) biopsies of muscle and adipose tissue can cause pain, bleeding, and infection**;** 4) magnetic resonance spectroscopy and MRI can be harmful for people with metal implants in their bodies, and may cause discomfort in people with claustrophobia; (those with implants or history of claustrophobia will be excluded); 5) if an arterial line is placed, a blood clot can occur at the site of the catheter insertion and could decrease blood flow to the hand causing tissue damage (however, the risk of this is extremely small because only a small size catheter is used and it is kept in for a short period of time; furthermore, before catheter placement, the hand is examined to ensure adequate blood flow); 6) the risks of infusing stable isotope tracers include the possibility of pyrogen response or infection; however, all solutions will be tested for pyrogens and sterility before infusion and will be administered under strict aseptic conditions; there are no known short- or long-term risks associated with the infusion of the isotopes themselves; 7) the effect of TUDCA on ART absorption and handling is not known therefore we will enrol only subjects with controlled HIV and will monitor CD4 counts and viral load upon completion of this brief study. These results will be reviewed by the DSMB to ensure patient safety.

Adequacy of protection against risks. All experimental procedures will be explained in writing and orally to the subject by the principal investigator, co-investigator, or the research coordinator. Written informed consent will be obtained after all questions have been answered and the risks and benefits of participation in the study have been described. The objectives of the project, all of the requirements for participation, and any possible discomforts and risks will be clearly explained to each subject orally and in writing in lay terms that he/she is able to comprehend. The subject must sign a consent form, approved by the Washington University School of Medicine Institutional Review Board, before participating in any aspect

of the study. To secure data transfer between WUMS and VUMS, a Secure File Transfer system will be used to transmit any PHI or study-related data. An original copy of each consent will be kept on file at the primary site with an additional signed consent at VUMS. At each site all data will be kept within a secure server and will be deidentified at the closure of the study.

H2 Compliance Program: During the intervention, subjects will be seen weekly by a member of the research team who will take weight, measure blood pressure and pulse, review any problems and verify compliance by using a pill count. Should the need for medical evaluation arise, they will be seen by the PI,Dr. Presti, Dr Hulgan, Dr. Koethe all of whom are certified in Internal Medicine.

Should the need for medical attention arise all the resources of a large teaching hospital at each site are available for subject evaluation and treatment. Complete physical examinations will be performed to screen out subjects who may be at potential risk. Subjects will be carefully monitored by the medical staff associated with the study for the duration of the study.

Potential benefits of the proposed research to the subjects and others. Potential benefits to the subjects participating in the study include information regarding their medical health, and screening for diabetes. Benefits to society include a better understanding of the factors responsible for insulin resistance in HIV and the mechanisms through which bile acids may improve these outcomes.

Importance of the knowledge to be gained. This study will provide important information regarding the causes of insulin resistance in HIV and will test the efficacy of a new modality. The risks associated with this research are minimal relative to the clinical implications of this study, which could lead to better therapies for preventing and treating the metabolic complications of PI-ART.

H3 Data and safety monitoring. Data from the study will be monitored on a continuous basis by the PI, co-investigators, study research nurse, and research coordinator. The PI, study research nurse, research coordinator, and postdoctoral research fellow will meet weekly to review any study issues. The PI, study research nurse, and research coordinator will review all serious adverse events (SAEs), adverse events (AEs), and laboratory values. Dr. Hulgan, the study research nurse and coordinator at VUMS will also discuss weekly eligible or enrolled subjects. In the event of an SAE or reportable event, Dr Hulgan or Koethe will contact Dr. Reeds within 24 hours. The Washington University Human Research Protection Office (HRPO) will be notified as a reportable event within 10 days of knowledge of the event. Follow-up will be performed as additional information becomes available. In addition, all laboratory values outside of normal range which place the participant at risk are discussed with the study participant and appropriate arrangements will be made for treatment, if necessary. Data from the study will be monitored on a continuous basis by the PI, co-investigators, research coordinator, and research study nurse.

A Data and Safety Monitoring Board, consisting of Shelby Sullivan, M.D. (Gastroenterologist), David Alpers. (Gastroenterologist), and David Clifford, M.D. (Infectious Diseases), will be available for discussion at any time with the PI to review any safety issues. All data will be treated confidentially and the subjects' names and identities will not be disclosed in any published reports. The subjects will be informed orally and in writing that they are free to withdraw from the study at any time with no bias or prejudice. The data obtained from this study will be kept confidential and no subjects will be identified in any written publications. Patients are assigned a study specific identifying number upon entry to the study, after which all medical information is referenced by this number. Clinic records are maintained in locked file cabinets within a locked room.

Appendix 1.

Animal Toxicity Studies (supplied by Bruschettini S.r.l.)

Name of company: BRUSCHETTINI S.r.l. Name of finished product: TAUROLITE Name of active ingredient: Tauroursodeoxycholic acid	TABULATED STUDY REPORT Ref. Part III B - Vol. 10			
REPEATED DOSE TOXICITY: (sub chronic toxicity)				
Ref. to document: prof. M. De Bernardi - University of Pavia - II Chair of Toxichology				
Bibliographic ref. (...):				
Species: Dog / Beagle			Number of animals: 18 (9 M + 9 F)	
Administration route: i.p. saline solution			Test article: TUDCA	
Study group	1	2	3	
Number of test animals	3 M + 3 F	3 M + 3 F	3 M + 3 F	
Dosage and frequency (mg/kg/day)	Saline solution	50 mg/kg/day	150 mg/kg/day	
Duration of treatment (weeks)	4	4	4	
TESTS Animal behaviour Blood chemistry: erythrocytes, heamoglobin, haematocrit, leucocytes with formula Biochemical: glycemia, azotemia, creatininemia, cholesterolemia, serum electrolytes (Na, K, Ca), bilirubine, protidemia, serum-electrophoresis, SGOT, SGPT, ALP Urine tests: measurement of volume in 24 hours, specific weight, protein, glucose, ketonic bodies, bilirubin, blood microscopic sediment test. Macroscopic test and weight of organs: liver, kdneys, spleen, surrenals, testicles, ovaries, heart, brain, pituitary gland, thymus and lungs Histology: lilver, kidneys, spleen, surrenals, testicles, ovaries, uterus, heart, brain, pituitary glands lungs, stomach, small intestine, colon, pancreas, bladder, thyroid, eye				
FINDINGS Biochemical and blood chemistry: 50 mg/kg/day - No pathological modification of the various parameters. 150 mg/kg/day - No pathological modification of the various parameters Behaviour - Macroscopic test - histology: 50 mg/kg/day - No behavioral, macroscopic, nor histological modification; 150 mg/kg/day - No behavioral, macroscopic, nor histological modification				

Name of company: BRUSCHETTINI S.r.l. Name of finished product: TAUROLITE Name of active ingredient: Tauroursodeoxycholic acid	TABULATED STUDY REPORT Ref. to Part III B Vol. 10	

REPEATED DOSE TOXICITY (chronic toxicity):
Ref. to document: Prof. M. De Bernardi
Bibliographic ref. (...):

Species: Rat / Sprague Dawley	Number of animals: 80 (40 M + 40 F)
Administration route: per os	Test article: TUDCA

Formulation of test substance and concentration: suspension in arabic gum 10% solution

Study group	1		2		3		4	
Number of test animals	10 M	10 F	10 M	10 F	10 M	10 F	10 M	10 F
Dosage and frequency (mg/kg/day)	arabic gum	placebo	300 mg/kg/day		600 mg/kg/day		1200 mg/kg/day	
Duration of treatment (weeks)	26	26	26	26	26	26	26	26

TESTS
Behaviour and body weight
Blood chemistry: erythrocytes, haemoglobin, haematocrit, leucocytes with formula
Biochemical: glycemia, azotemia, creatininemia, cholesterolemia, serum electrolytes (Na, K, Ca), bilirubinemia, protidemia sero-protein electrophoresis, SGOT, SGPT
Urine tests: measurement of volume in 24 hours, specific weight, pH, protein, glucose, ketonic bodies, bilirubin, blood, microscopic sediment test
Macroscopic test and weight of organs: liver, kidneys, spleen, surrenals, testicles, ovaries, uterus, heart, pituitary gland, thymus and lungs
Histology: liver, kidneys, spleen, surrenals, testicles, ovaries, uterus, heart, brain, pituitary gland, thymus, lungs, stomach, small intestine, colon, pancreas, bladder, prostate, thyroid, eye

FINDINGS
Biochemical and blood chemistry:
300 mg/kg/day no pathological modification of the various parameters;
600 mg/kg/day no pathological modification of the various parameters
1200 mg/kg/day no pathological modification of the various parameters
Behaviour - Macroscopic test - Histology
300 mg/kg/day no behavioural, macroscopic nor histological modification
600 mg/kg/day no behavioural, macroscopic nor histological modification
1200 mg/kg/day no behavioural, macroscopic nor histological modification

Name of company: BRUSCHETTINI S.r.l. Name of finished product: TAUROLITE Name of active ingredient: Tauroursodeoxycholic acid	TABULATED STUDY REPORT Ref. to Part III B Vol. 12	

REPEATED DOSE TOXICITY (chronic toxicity):								
Ref. to document: Prof. F.A. Bertè								
Bibliographic ref. (...):								
Species: rat / Winstar				Number of animals: 80 (40 M + 40 F)				
Administration route: p.o. suspension in gum arabic 10%				Test article: TUDCA				
Study group	1		2		3		4	
Sex (m/f)	m	f	m	f	m	f	m	f
Number of test animals	10	10	10	10	10	10	10	10
Dosage and frequency (mg/kg/day)	placebo		250 mg/kg/day		500 mg/kg/day		1000 mg/kg/day	
Duration of treatment (weeks)	26	26	26	26	26	26	26	26

TESTS
Mortality, behaviour, body weight, water and food consumption
Blood chemistry: erythrocytes, haemoglobin, haematocrit, leucocytes with formula
Biochemical: glycemia, azotemia, protidemia, cholesterolemia, serum electrolytes (Na, K, Ca), bilirubinemia, SGOT, SGPT, ALP
Urine tests: protein, glucose, ketonic bodies, bilirubin, blood
Weight of organs: brain, thyroid, heart, liver, kidneys, spleen, testicles, ovaries
Macroscopic test and histological tests: brain, heart, thyroid, liver, lungs, kidneys, spleen, surrenals, testicles, ovaries, lymphatic ganglions, salivary glands, mammary glands, pituitary gland, thymus, pancreas, bladder, prostate, uterus, eyes, spinal marrow, gastroenteric apparatus (trachea, oesophagus, stomach, small intestine, colon, ribs, femurs, vertebrae (including bone marrow)

FINDINGS
Biochemical and blood chemistry:
250 mg/kg/day no pathological modification of the various parameters;
500 mg/kg/day no pathological modification of the various parameters
1000 mg/kg/day no pathological modification of the various parameters
Behaviour - Macroscopic test - Histology
250 mg/kg/day no behavioural, macroscopic nor histological modification
500 mg/kg/day no behavioural, macroscopic nor histological modification
1000 mg/kg/day no behavioural, macroscopic nor histological modification

Tauroursodeoxycholic acid for protease-inhibitor associated insulin resistance
PI: Reeds

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