Triglyceride: High-Density Lipoprotein Cholesterol Effects in Healthy Subjects Administered a Peroxisome Proliferator Activated Receptor δ Agonist

Dennis L. Sprecher, Christine Massien, Greg Pearce, Andrew N. Billin, Itay Perlstein, Timothy M. Willson, David G. Hassall, Nicolas Ancellin, Scott D. Patterson, David C. Lobe, Tony G. Johnson

Objectives—Exercise increases fatty acid oxidation (FAO), improves serum high density lipoprotein cholesterol (HDLc) and triglycerides (TG), and upregulates skeletal muscle peroxisome proliferator activated receptor (PPAR)δ expression. In parallel, PPARδ agonist-upregulated FAO would induce fatty-acid uptake (via peripheral lipolysis), and influence HDLc and TG-rich lipoprotein particle metabolism, as suggested in preclinical models.

Methods and Results—Healthy volunteers were allocated placebo (n=6) or PPARδ agonist (GW501516) at 2.5 mg (n=9) or 10 mg (n=9), orally, once-daily for 2 weeks while hospitalized and sedentary. Standard lipid/lipoproteins were measured and in vivo fat feeding studies were conducted. Human skeletal muscle cells were treated with GW501516 in vitro and evaluated for lipid-related gene expression and FAO. Serum TG trended downwards (P=0.08, 10 mg), whereas TG clearance post fat-feeding improved with drug (P=0.02). HDLc was enhanced in both treatment groups (2.5 mg P=0.004, 10 mg P<0.001) when compared with the decrease in the placebo group (~11.5±1.6%, P=0.002). These findings complimented in vitro cell culture results whereby GW501516 induced FAO and upregulated CPT1 and CD36 expression, in addition to a 2-fold increase in ABCA1 (P=0.002). However, LpL expression remained unchanged.

Conclusions—This is the first report of a PPARδ agonist administered to man. In this small study, GW501516 significantly influenced HDLc and TGs in healthy volunteers. Enhanced in vivo serum fat clearance, and the first demonstrated in vitro upregulation in human skeletal muscle fat utilization and ABCA1 expression, suggests peripheral fat utilization and lipiddation as potential mechanisms toward these HDLc:TG effects. (Arterioscler Thromb Vasc Biol. 2007;27:359-365.)

Key Words: lipid ■ PPAR ■ cholesterol ■ triglyceride

Peroxisome proliferator-activated receptors (PPARs) are a family of nuclear receptors designated PPARα (NR1C1), PPARβ/δ (NR1C2), and PPARγ (NR1C3) which serve as transcription factors.1,2 PPARs control the cascade of fatty acid intracellular metabolism. Although agonists of the PPARα (fibrates) and γ isoforms (thiazolidinediones) are currently marketed, a specific PPARδ agonist has not heretofore been admiistered to human subjects. Preclinical animal models have suggested that PPARγ stimulation promotes fat storage in cells, particularly in adipose tissue where this PPAR is especially abundant.3,4 In contrast, activation of PPARα and δ enhance fatty acid oxidation (FAO), leading to increased energy production and possible uncoupling. Both PPARα and δ activation have similar transcriptional effects on the genes associated with fat cellular uptake (eg, the fatty acid translocator, CD36/FAT) and metabolism (eg, carnitine palmitoyl transferase-1, CPT1), but their tissue specificity differs. PPARδ is ubiquitously expressed, in contrast to PPARα which has substantial activity in the liver. Muscle contains both PPARα and δ, although PPARδ appears to predominate.5,6

It has been reported that exercise mediates upregulation of PPARδ and creates a requirement for an external (or serum-derived) triacylglycerol energy source. Lipoproteins are the major source of such combustible substrate, thereby suggesting the interplay between PPAR activity and lipoprotein modification. Mediation of this lipoprotein:cell transfer of lipid is processed through lipolytic activity, and influenced by...
scavenger receptor B1 (SR-B1) and ATP-binding cassette transporter protein-A1 (ABCA1) mechanisms. These mechanisms could be responsible for the changes in HDLc and TG concentrations observed with physical activity.6 HDLc is known to increase with exercise, and there is a tendency for HDLc to decrease with a drop in physical activity, the latter perhaps based on a similar mechanism.9 In support of this hypothesis, Tikkanen et al identified a striking correlation between increased carnitine palmitoyltransferase activity (CPT1; a protein that transports fats across the mitochondrial membrane) and elevated HDLc in the setting of exercise.10 Potentially, the administration of a synthetic PPARδ agonist can induce an “exercise-like” alteration in HDL. Consistent with this proposal, PPARδ-directed changes in the skeletal muscle of mice result in marked increases in exercise tolerance.11

The data presented here represent the first in vivo experimental human data reported with a PPARδ agonist. We demonstrate an enhanced HDLc response, as well as TG metabolism compared with placebo. Additionally, our findings suggest upregulated fat utilization/clearance as responsible, in part, for these changes.

Methods

Clinical Study Design and Efficacy End Points

Healthy white normolipidemic male volunteers were randomly allocated placebo (n = 6) or GW501516 in one of two doses: 2.5 mg (n = 9) or 10 mg (n = 9), orally, once-daily for 2 weeks during hospitalized care. GW501516 is a potent (EC50 of 1.2 nm ±0.1 nmol/L) and specific PPARδ agonist in vitro (>1200-fold selective for PPARδ relative to α and γ).12-14 Therefore, the doses of GW501516 used in this study did not crossover to PPARα or PPARγ. During this study, subjects were sedentary and prohibited from performing rigorous physical activity. They received a diet of approximately 2000 kcal and 62.6 g of fat (26% of total calories) per day. Safety and tolerability were evaluated through adverse events, vital signs, 12-lead ECG telemetry, and clinical laboratory measurements. Standard lipids/ lipoproteins were measured and analyzed after an overnight fast before, during, and after the 14-day dosing period. GW501516 plasma levels were measured on Days 10, 12, and 14 (predose) and at designated times on day 14. The study was conducted at Northwick Park Hospital, Harlow, UK. Written informed consent was obtained before each subject could participate in the study in accordance with the relevant Institutional Ethics Committee. The study was conducted in accordance with “good clinical practice” (GCP) and all applicable regulatory requirements, including the 1996 version of the Declaration of Helsinki and the Institutional Ethics Committee.

Total plasma cholesterol and TG concentrations were determined by enzymatic methods using a Hitachi 917 Biochemical Analyzer (Hitachi Ltd, Japan). HDLc was measured enzymatically (Boehringer Mannheim). Low-density lipoprotein cholesterol (LDLc) was calculated by the Friedewald equation. Apolipoprotein AI (apoAI) concentrations were determined by immunonephelometry (Dade Behring BNII Nephelometer); interassay CVs were <4.3%.

A fat feeding study was conducted in which subjects were fed a high-fat (20 g) breakfast on Days 0 and 13 of the study at 1 hour post-dose. Subjects were fasted for more than 10 hours before receiving the meal. Subjects received a low fat lunch, dinner, and snack at 4, 10, and 14 hours post-dose, respectively. Blood samples were collected at 0, 1, 2, 3, 4, 6, 8, 10, 12, 15, and 24 hours post-fat feeding for TG measurements. TG analysis was performed as described above.

Fatty Acid Oxidation and Gene Expression Analyses in Human Skeletal Muscle Cells

Human skeletal muscle (HSKM) cells were purchased from Cambrex BioScience (Walkersville, Md). Cells were plated at 100 000 cells per well in a 12-well Costar plate and dosed in DMEM, 10% FBS with 10 nmol/L GW501516 or DMSO (vehicle control) for 2 days. This dose of GW501516 was chosen to provide the maximum response based on published dose-response experiments.14,15 The day before harvesting for gene expression or FAO analysis, cells were re-fed with the same media along with fresh compound. FAO assays were performed as described16 (see supplemental Methods, available online at http://atvb.ahajournals.org).

Gene expression analyses were performed as described.17 Total RNA was isolated using the Promega SV RNA isolation system according to the manufacturer’s instructions. Real-time quantitative polymerase chain reaction was performed using an ABI Prism 7700 sequence detection system instrument and software (Applied Biosystems, Inc). Gene-specific primers and probes were designed using Primer Express Version 2.0.0 (Applied Biosystems, Inc) and synthesized by Keystone Laboratories (supplemental Table I and Methods).

Statistical Analyses

Lipid response data were evaluated for within group changes using paired t tests and between group differences using unpaired t tests. Change from baseline (CBF) values for the treatment groups were compared with placebo using mixed models in SAS (V9.1, SAS Institute) accounting for baseline lipid value as a covariate. For the fat feeding study, TG concentrations were measured over 24 hours after fat feeding at Days 0 and 13. Maximum TG values (TGmax) and area under the TG curve (TG-AUC) were calculated at each time period. Correlations between change in TG-AUC and serum TG were evaluated with simple Pearson correlation coefficients followed by analysis of variance models adjusting for baseline TG.

Results

Clinical Results

The mean age of study subjects was 30 ± 5 years, and mean body mass index was 24 ± 3 with no significant between-group differences for either measure. GW501516 was safe and well-tolerated. Subjects treated with GW501516 had no significant adverse effects, including liver and muscle responses. We measured liver enzymes (total bilirubin, alkaline phosphatase, aspartate transaminase, alanine transaminase) and muscle proteins (CPK, aldolase, creatinine) for any changes associated with GW501516 treatment. None were noted. Steady-state was considered to have been achieved by two weeks, with GW501516 plasma concentration (Ceq max) ranging between 33 ng/mL to 88 ng/mL at the 2.5 mg dose and between 110 ng/mL to 327 ng/mL at the 10 mg dose. The Table shows lipid responses over the study period.

TG levels declined significantly in the 2.5 mg (−15.4 ± 6.5%, P = 0.046) and 10 mg (−23.4 ± 7.0%, P = 0.01) treatment groups, while staying relatively stable in the placebo group (+6.1 ± 10.2%, P = 0.84). These TG responses in the treatment groups, however, were not significantly different than placebo after adjustment for baseline TG (P = 0.16 for 2.5 mg; P = 0.08 for 10 mg).

Contrary to TG, HDLc showed a significant decrease in the placebo group (−11.5 ± 1.6%, P = 0.002), while showing less movement in the two treatment groups (2.5 mg: 1.8 ± 2.6%, P = 0.43; 10 mg: 5.3 ± 2.7%, P = 0.12). However, compared with placebo, the HDLc response was significantly enhanced in both the 2.5 mg (P = 0.004) and 10 mg (P < 0.001) groups when adjusted for baseline HDL (Figure 1). The stability of
TABLE 1. Lipid Profiles and Percent Change From Baseline (CFB) Over the Course of Study by Treatment Group

<table>
<thead>
<tr>
<th>Group</th>
<th>Baseline (mmol/L)</th>
<th>End of Study (mmol/L)</th>
<th>Percent CFB</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC–Placebo</td>
<td>4.18±0.32</td>
<td>3.92±0.20</td>
<td>−4.5±5.5</td>
</tr>
<tr>
<td>2.5 mg</td>
<td>3.74±0.17</td>
<td>3.71±0.20</td>
<td>0.3±7.4</td>
</tr>
<tr>
<td>10 mg</td>
<td>4.39±0.23</td>
<td>4.04±0.18</td>
<td>−7.1±3.4</td>
</tr>
<tr>
<td>LDLc–Placebo</td>
<td>3.01±0.36</td>
<td>2.88±0.22</td>
<td>−2.5±0.02</td>
</tr>
<tr>
<td>2.5 mg</td>
<td>2.38±0.14</td>
<td>2.45±0.18</td>
<td>2.5±0.02</td>
</tr>
<tr>
<td>10 mg</td>
<td>2.96±0.22</td>
<td>2.68±0.18</td>
<td>−7.6±5.8</td>
</tr>
<tr>
<td>HDLc–Placebo</td>
<td>1.12±0.05</td>
<td>0.99±0.05</td>
<td>−11.5±1.6</td>
</tr>
<tr>
<td>2.5 mg</td>
<td>1.04±0.06</td>
<td>1.06±0.08</td>
<td>1.8±2.6</td>
</tr>
<tr>
<td>10 mg</td>
<td>1.16±0.06</td>
<td>1.22±0.05</td>
<td>5.3±2.7</td>
</tr>
<tr>
<td>TG–Placebo</td>
<td>0.72±0.09</td>
<td>0.75±0.10</td>
<td>6.1±10.2</td>
</tr>
<tr>
<td>2.5 mg</td>
<td>0.93±0.08</td>
<td>0.77±0.07</td>
<td>−15.4±6.5</td>
</tr>
<tr>
<td>10 mg</td>
<td>1.04±0.15</td>
<td>0.76±0.09</td>
<td>−23.4±7.0</td>
</tr>
<tr>
<td>ApoAI–Placebo</td>
<td>111±4</td>
<td>102±5</td>
<td>−7.8±2.3</td>
</tr>
<tr>
<td>2.5 mg</td>
<td>111±4</td>
<td>107±4</td>
<td>−3.2±1.6</td>
</tr>
<tr>
<td>10 mg</td>
<td>116±4</td>
<td>116±3</td>
<td>−0.1±2.5</td>
</tr>
</tbody>
</table>

*P<0.01 for within group change; †P<0.01 vs Placebo; ‡P<0.05 for within group change; §P<0.05 vs Placebo.

Apolipoprotein A1 concentration for the 10 mg group represented a 7.7% improvement compared with placebo (P=0.03). Baseline systolic blood pressure values did not differ significantly among groups (Placebo=110±2 mm Hg, 2.5 mg=118±4 mm Hg, 10 mg=122±4 mmHg; P=0.16), nor did baseline diastolic blood pressure (Placebo=65±2 mmHg, 2.5 mg=63±2 mm Hg, 10 mg=68±2 mm Hg; P=0.17). There were no significant changes over the course of the study for systolic (Placebo P=0.10, 2.5 mg P=0.90, 10 mg P=0.81) or diastolic (Placebo P=0.48, 2.5 mg P=0.63, 10 mg P=0.70) blood pressures.

Fat Feeding Study

Lipolytic activity was gauged with TG-AUC calculations after fat feeding (Figure 2). The placebo group showed an increase of 36.1±14.6% in TG-AUC from baseline to end, whereas the 2.5 mg group decreased 15.3±9.8% (P versus placebo=0.07), and the 10 mg group decreased 25.4±11.2% (P versus placebo=0.01). It should be noted that central tendency is difficult to estimate in the placebo group because 4 subjects experienced increases of greater than 40%, 1 had an increase of 13%, and 1 had a decrease of 24%. However, parametric and nonparametric statistical approaches yielded the same conclusions with regard to treatment effects. Change from baseline in TGmax showed similar patterns with an increase of 17.2±15.5% for the placebo group compared with −8.8±8.8% (P=0.09) for the 2.5 mg group and −13.4±7.8% (P=0.03) for the 10 mg group. Adjustments for baseline TG levels did not alter the conclusions.

The percentage of patients with decreases in TG-AUC follows a dose-response paradigm (P for trend=0.02). Moreover, the change in TG-AUC was significantly correlated to the change in serum TG for patients on active treatment (r=0.60, P=0.009). Although correlation analyses are prone to Type II error in small studies, neither baseline TG nor end TG correlated significantly with TG-AUC change in active treatment subjects. No significant correlations were noted in the placebo group.

**Effects of GW501516 on Gene Expression and Fatty Acid Oxidation in Human Skeletal Muscle Cells**

The mRNA levels of FAO and lipid handling genes were determined in HSKM cells treated with 10 nmol/L GW501516 (Figure 3). GW501516 induced CD36 mRNA expression approximately 2-fold (P=0.049) above the vehicle (Figure 3A). Further, the genes encoding CPT1a and CPT1b are both expressed in HSKM cells and both respond to GW501516. CPT1a was induced nearly 5-fold (P=0.005; Figure 3B) and CPT1b nearly 3-fold (P=0.02; Figure 3C) above the vehicle. We determined that although the pyruvate dehydrogenase kinase-4 (PDK4) gene is barely detectable in vehicle-treated HSKM cells, it is induced approximately 200-fold (P=0.003) in response to GW501516 (Figure 3D). To determine whether GW501516 regulates fatty acid combustion, a FAO assay was performed, revealing that GW501516 induced oleate oxidation approximately 7-fold (P<0.001) compared with vehicle (Figure 4). We routinely checked that the HSKM cells remained viable throughout the studies and that cell toxicity did not occur. No reduction in cell viability was noted (data not shown).
Further, to determine whether genes involved in HDL formation and flux are regulated by GW501516, we measured the quantity of LpL, SR-B1, ABCG1, and ABCA1 mRNA transcripts. Whereas LpL, SR-B1, and ABCG1 were expressed in HSKM cells, none were regulated by GW501516 (data not shown). Also, GW501516 had no effect on mRNA expression of PPARα, PPARγ, or PPARδ (data not shown). In contrast, ABCA1 was induced approximately 2-fold (P<0.002) compared with vehicle (Figure 3E). Finally, enhanced cholesterol efflux (30%, P=0.001) was observed when GW501516 (100 nM) was added to cholesterol-loaded HSKM cells and whole serum was used as an acceptor (supplemental Figure I).

Discussion

The PPARδ agonist-induced lipoprotein effects (eg, an increase in HDLc of up to 16.8%) were observed as the dose increased to 10 mg of GW501516. Closer inspection of the placebo cohort within this small trial revealed the physiologically expected reduction in HDLc (−11.5%, P=0.002) when requiring the study participants to be in-house and sedentary. Previous preclinical studies demonstrated that administration of GW501516 in obese rhesus monkeys at doses of 0.6 and 2.0 mg/kg/d produced a significant increase in HDLc and a reduction in TG values at similar GW501516 plasma exposure levels observed in our human experiment at daily doses of 2.5 mg and 10.0 mg, respectively. The most dramatic results observed in these animals were at the 6.0 mg/kg/d, with exposure level considerably above those observed in humans at the 10.0 mg dose. These pharmacodynamic (PD) results were recently corroborated by similar findings in Vervet monkeys administered with 3.0 mg/kg/d dose of GW501516, leading to over 3-fold higher exposure level than observed in human at the 10.0 mg dose. However, the pathophysiologic mechanisms for these changes remain unclear. The comparable HDL:TG modifications with both exercise and PPARδ agonism are conducive to theories surrounding lipolytic activity.

When placebo subjects were administered a fat meal, a substantial increase in post-fat feeding TG-AUC was observed after the 2 weeks of inactivity, consistent with a reduction in lipolytic activity, and in turn a reduction in tissue uptake of fat. Such a clinical assay has been well correlated with LpL activity assessed after heparin infusion. As such, these kinetic assays for LPL activity have been associated with circulating TG and HDLc concentrations. In contrast to the placebo group, the 2.5 mg and, significantly, the 10 mg dose of GW501516 reduced the post-fat feeding TG-AUC and maximum TG levels, suggesting amelioration of the lipolytic deficiency induced by a sedentary status. Reduction in lipolysis has been reported to be associated with lack of muscle movement. In rodent studies, there is a dramatic (10- to 20-fold) and rapid (initiating within 4 hours) decrease in LpL activity (with no change in mRNA) when muscle contraction is severely limited, particularly related to red-oxidative muscle. Stabilization of the HDL particle, through such lipolytic processing, leading to a reduced fractional catabolic rate is consistent with a longer HDL half-life reported in physically active men, and progressively higher apoAI levels in exercise-trained individuals of advancing physical activity groups.

There are, however, other explanations for these observations. The in-house–provided low fat meals may have played a role in reducing HDLc within the placebo group. The positive association between saturated fat intake and HDLc is well documented. Azrolan et al have proposed that the hepatic-lipid cellular milieu may sequester translatable mRNA, and/or modify translation initiation sites, thereby depressing apoAI synthesis when the environment is saturated-fat deficient. Further, there are 3 alternate ABCA1 transcripts, and the expression of these transcripts is tissue-specific and varies in response to diet. Preclinical models suggest modest PPARδ activity in liver to counter these
changes, notwithstanding secondary effects imposed from the periphery. In addition, PPAR\(\gamma\) is expressed in the intestine and may have influenced fat absorption. Preclinical models have suggested a reduction in cholesterol absorption. However, whereas long chain fatty acid intestinal uptake may be modified, it has been reported that PPAR\(\gamma\) activation does not affect total fat absorption in rodent models. Lack of a weight loss trend and no reported stool abnormalities also lowers the probability of this option. Yet, although we propose lipolysis as contributory to the HDL/TG effects, we cannot exclude other PPAR\(\gamma\)-mediated mechanisms.

The association between PPAR\(\gamma\)-induced TG reductions and TG-AUC values after a fat feeding in our study with active treatment is consistent with the lipolysis concept. In contrast, there was no association between TG-AUC and the observed HDLc benefits. Although these cohorts are small, and cannot be used to exclude a particular mechanistic pathway, these data could suggest that the PPAR\(\gamma\)-mediated HDLc change does not depend on lipolysis to the same extent as that of changes in TG or requires an alternative explanation.

In both the published obese rhesus monkey and HSKM cell gene analyses reported herein, ABCA1 expression was found to be upregulated by a PPAR\(\gamma\) agonist. ABCA1 protein–deficient subjects cannot properly lipidate lipid-poor apoAI particles, and are noted to have severely low HDLc values. PPAR\(\alpha\) and \(\gamma\) agonists upregulate ABCA1 gene expression. The extent to which peripheral PPAR\(\gamma\), (rather than hepatic) activation plays a role in enhancing ABCA1-mediated cholesterol efflux to HDL remains unclear.

Finally, changes in glucose tolerance (admittedly unlikely in healthy volunteer subjects) could have contributed to the

**Figure 3.** Effects of GW501516 on mRNA expression in HSKM cells. Cells were incubated with 10 nmol/L GW501516 and analyzed for gene expression by RTQ-PCR. Data were normalized to 36B4. Normalized values were an average of 3 biological replicates and presented as copies of target RNA. A, CD36; \(P=0.049\). B, CPT1a; \(P=0.005\). C, CPT1b; \(P=0.021\). D, PDK4; \(P=0.003\). E, ABCA1; \(P=0.002\).
observed TG and HDL changes. Lee et al and Tanaka et al have noted an improvement in insulin sensitivity when a PPARα agonist, including GW501516, is administered to rodents. This is hypothesized to be associated with reductions in the pool of skeletal muscle fat, a feature relevant to insulin resistance. In our 2-week clinical-safety trial reductions in the pool of skeletal muscle fat, a feature relevant with SEM.

$P$ increase in FAO with human exercise and associated to plasma proteins (data not shown). The parallel documented to be much lower because of the high binding of GW501516. GW501516 concentrations far below the plasma levels observed. Nonetheless, free concentration circulating levels (available to the target tissues) are expected to be much lower because of the high binding of GW501516 to plasma proteins (data not shown). The parallel documented increase in FAO with human exercise and associated upregulation of the binding and transport fatty acid proteins (CD36/FAT, FABP), mitochondrial carrier protein CPT1, and the counter-regulatory PDK. In human muscle biopsy tissue prompts speculation that exercise-induced FAO is mechanistically generated via a PPARδ physiological switch. Such a concept is consistent with upregulation of PPARδ shortly after exercise initiation in humans.

There are caveats to the outcomes presented in this report. The increase in HDLc of 16.8% at the 10 mg dose is predicated on a reduction in placebo of 11%. Until larger studies and/or those of longer duration are performed with stable outpatient placebo populations, the actual HDLc benefits cannot be defined. Further, although nursing logs and protocol design requirements defined a sedentary bed-ridden existence for 2 weeks, we did not directly quantify physical activity. We do know, however, that these individuals did not leave the small research unit within the study time interval of two weeks. In addition, diet-log estimates of fat intake (26% of total calories) were assumed to be lower than baseline, even though the entry dietary patterns were not interrogated. Although this study does not perfectly support an exercise pathway, it does provide insight toward HDLc values in the resting state. To investigate thoroughly the impact of GW501516 treatment on HDL levels in the setting of exercise, both resting and nonresting subjects need to be evaluated in parallel and muscle biopsies evaluated for relevant genes of interest. However, this small study was designed to study the effects of a repeat-dose of GW501516 in healthy subjects. Additional studies that address these issues are planned.

In conclusion, the results of this study provide support for the influence of PPARδ on the enzymes critical to FAO in human cells, comparable to those observed in murine and monkey models, and similar to that observed with exercise. Further, HDLc and TG responses observed in this first use of a PPARδ agonist in human subjects were similar to lipid responses in preclinical models. We hypothesize that GW501516-induced enhancement of fatty-acid utilization contributes to these lipoprotein changes.

**Disclosures**

All authors have employment and ownership interest in GlaxoSmithKline.

**References**

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SUPPLEMENTAL MATERIAL

Triglyceride: HDLc Effects in Healthy Subjects Administered a Peroxisome Proliferator Activated Receptor (PPAR) δ Agonist.

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Supplemental Methods

Determination of GW501516 Plasma Concentrations: GW501516 plasma concentrations were determined by validated analytical methods based on acetonitrile protein precipitation, followed by HPLC/tandem mass spectrometry. Briefly, 2 ml of whole blood was collected at specified times into lithium heparin tubes, mixed and centrifuged for 15 minutes at 4°C, 1500 g. Plasma (1 ml) was stored at -20°C pending analysis. Plasma samples (30 ul) and an internal standard in acetonitrile (120 ul) were aliquoted together and extracted by 96-well protein precipitation using a MultiProbe® II liquid handling system. Water (150 ul) was added to the extracts, and then mixed and sealed. The samples were analyzed via HPLC/ tandem mass spectrometry (LC/MS/MS) using selected reaction monitoring (SRM). GW501516 plasma concentration-time data were analyzed by non-compartmental methods using WinNonlin Professional Version 4.1.

Fatty Acid Oxidation Assay: HSKM cells were incubated at 37°C in 12- or 24-well plates containing 500 or 750 ul of low serum differentiation medium (DMEM containing 2% horse serum, 4 mM glutamine, 50 mg/ml gentamycin) plus 12.5 mM HEPES, 0.25% bovine serum albumin, 1.0 mM carnitine, 100 uM sodium oleate, 50 mg/ml gentamycin, and 1.0 uCi/ml [14C]oleate (PerkinElmer Life Sciences). After 3 h, the incubation medium was transferred to new dishes and assayed for labeled oxidation products (CO₂ and acid-soluble metabolites). The cells were washed with PBS, lysed with 200 ul of M-PER buffer (Pierce) and the amount of protein/well determined using BCA reagent (Pierce).
Gene Expression Analyses: All primers and probes were entered into the NCBI BLAST program to ensure specificity. Fold induction values were calculated as the ΔCt (difference in cycle threshold values for vehicle and treatment and represented as transcript number) after normalizing each biological replicate to the calculated transcript quantity for 36B4. The normalized values were averaged for the three biological replicates and SEM calculated. Values are presented as copies of target RNA divided by copies of 36B4. 36B4 values did not deviate by more than 2-fold in any given sample.

Cholesterol Efflux Assay in Human Skeletal Muscle Cells: 100,000 HSKM cells were plated in 24-well dishes and allowed to attach for 16 hours. Cells were labeled in DMEM with 5uM mevastatin, 100uM mevalonic acid, 10% lipoprotein-depleted serum, and 5uCi per ml of tritiated cholesterol along with ligands for 16 hours. The cells were then washed in serum-free medium and incubated for 24 hours in medium supplemented with 5% whole serum and test ligands. Test ligands were GW501516 (100nM) and the LXR agonist T1317 (1uM, Tularek) as a comparator. Mevastatin and mevalonate were not added at this step. At the end of the 24 hour period supernatants were recovered and cell debris pelleted prior to scintillation counting. Cells were washed and lysed with isopropanol and an aliquot was counted to determine labeling efficiency.
Supplemental Table I: Primer Sequences used for mRNA Expression Analyses

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<th>Forward</th>
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Sprecher DL et al.
Supplemental Figure I. Effect of GW501516 on Cholesterol Efflux in Cultured Human Skeletal Muscle Cells.

Supplemental Figure I Legend. Effect of GW501516 on Cholesterol Efflux in Cultured Human Skeletal Muscle Cells. HSKM cells were treated with 100nM GW501516 or 1uM T1317 (an LXR agonist) using whole serum as an acceptor as described in the Methods. The assay was performed in triplicate and data presented as mean fraction of total effluxed labeled cholesterol (bottom solid bar) with SD (stacked upper open bar). GW501516 increased efflux ~1.3 fold over vehicle (p=0.001). As a comparison, the LXR agonist T1317 increased efflux about 2 fold.