Ertiprotafib Improves Glycemic Control and Lowers Lipids Via Multiple Mechanisms

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ABSTRACT

Ertiprotafib belongs to a novel class of insulin sensitizers developed for treatment of type 2 diabetes. In insulin resistant rodent models, ertiprotafib and a close analog lowered both fasting blood glucose and insulin levels, and improved glycemic excursion during an oral glucose tolerance test. Additionally, treatment of rodents improved lipid profiles, with significantly lowered triglyceride and free fatty acid levels. These results suggested that this therapeutic activity might involve mechanisms in addition to PTP1b inhibition. Here, we demonstrate that ertiprotafib activates PPARα and PPARγ at concentrations comparable to known agonists of these regulators. Furthermore, it is able to drive adipocyte differentiation of C3H10T1/2 cells, a hallmark of PPARγ activation. Livers from ertiprotafib treated animals showed significant induction of acyl-CoA oxidase activity, likely due to PPARα engagement in these animals. We also show that ertiprotafib inhibits PTP1b in vitro with non-classical kinetics at concentrations above its EC50s for PPAR agonism. Thus, the complete mechanism of action for ertiprotafib and related compounds in vivo may involve multiple independent mechanisms, including (but not necessarily limited to) PTP1b inhibition and dual PPARα/PPARγ agonism. Ertiprotafib pharmacology and interpretation of clinical results must be seen in light of this complexity.
Introduction

Increasing prevalence of obesity and type 2 diabetes continues to drive the search for better pharmacologic treatments for dyslipidemia and hyperglycemia (Inzucchi, 2002; Flier, 2004). Thiazolidinediones provide a first opportunity to improve the underlying insulin resistance in patients, through activation of the peroxisome proliferator activated receptor-γ (PPARγ) (Willson et al., 2000; Rosen and Spiegelman, 2001). These agents regulate gene transcription in insulin-sensitive tissues, resulting ultimately in reduced glucose levels in patients through increased utilization in skeletal muscle and decreased de novo synthesis in the liver (Willson et al., 2001; Way et al., 2001). Improvements to TZD therapy now focus on improving glycemic and lipid control while avoiding the weight gain and edema seen with these agents (Inzucchi, 2002; Nesto et al., 2003; Diamant and Heine, 2003).

One approach that is receiving considerable attention is to combine PPARγ agonism with activation of PPARα to create a dual agonist (Auwerx, 1999; Murakami et al., 1998; Lohray et al., 2001; Liu et al., 2001; Cronet et al., 2001; Brooks et al., 2001). The fibrate class of lipid-lowering drugs act as agonists of PPARα to regulate fatty acid metabolism and ketogenesis in the liver (Willson et al., 2000; Kersten et al., 2000). These potent hypolipidaemic agents are used to lower plasma triglyceride levels and raise high-density lipoprotein (HDL) levels. Thus, combining agonism for these receptors in a dual PPARα/γ ligand could provide clear advantages to treat the hyperglycemia and dyslipidemia in diabetic patients that have an increased risk for cardiovascular disease (Brooks et al., 2001).

Targeting the protein tyrosine phosphatase PTP1b also provides an opportunity to improve insulin sensitivity without the weight gain seen with current PPARγ activators. Genetically deficient PTP1b knock out mice remain healthy with sustained phosphorylation of the insulin receptor, which results in profound insulin sensitivity with improved glucose
tolerance, and lower levels of circulating insulin (Klaman et al., 2000; Elchebly et al., 1999). Moreover, these mice have lower adiposity and are resistant to weight gain due to their increased sensitivity to leptin (Zabolotny et al., 2002; Cheng et al., 2002). Additionally, mice treated with PTP1b anti-sense oligonucleotides show increased insulin-dependent signaling, resulting in blood glucose normalization and improved insulin sensitivity (Zinker et al., 2002; Gum et al., 2003). Thus, inhibition of PTP1b could prolong insulin receptor phosphorylation, improve insulin action, normalize glucose levels, and reduce adiposity in diabetic patients.

Previously, members of a class of novel arylbenzonaphthothiophenes and arylbenzonaphthofurans were developed as PTP1b inhibitors and shown to improve insulin sensitivity in rodents (Wrobel et al., 1999). One compound from these efforts, ertiprotafib, progressed to Phase II clinical trials for treatment of type 2 diabetes. However, data from in vivo treatment of rodents with ertiprotafib were inconsistent with PTP1b inhibition as the sole mechanism for these changes. Notably, bone marrow hypocellularity due to adipocyte deposition was observed in rats treated with supra-pharmacologic doses of ertiprotafib, an indication of possible PPARγ activation. Furthermore, treated rats showed moderate dose-related increases in liver weights along with ultra-structural signs of peroxisome proliferation, implying potential PPARα activation (Peterson et al., 2004; Tong et al., unpublished; Casciotti et al., unpublished). These observations prompted the current investigations into the mechanism of action of ertiprotafib.

In this study, we show that ertiprotafib robustly increases insulin sensitivity in both a mouse and rat model of insulin resistance. In addition to effects on hyperglycemia and hyperinsulinemia, ertiprotafib and a close analog also dramatically reduce both serum triglyceride and free fatty acid levels in these insulin resistant rodent models. In vitro assays
indicated the likelihood that multiple independent pathways might contribute to the full pharmacological profile of ertiprotafib. Importantly, ertiprotafib was shown to inhibit PTP1b in a time dependent, enzyme concentration dependent manner not associated with classical, competitive inhibition. Additionally, PTP inhibition in cells occurred at relatively high (above 10 uM) concentrations. Further cellular studies indicated that ertiprotafib is at least a dual PPARα and PPARγ agonist with EC50s for transactivation near 1 uM, and is able to induce adipocyte differentiation of fibroblast cells, likely through PPARγ. Finally, livers from animals treated with ertiprotafib showed significant induction of the PPARα dependent enzyme, acyl-CoA oxidase. Taken together, these data indicate that ertiprotafib appears to use a complicated mechanism of action in vivo, involving at least engagement of PPARα and γ, in addition to possible PTP1b inhibition, to improve insulin sensitivity and lower lipids.
Materials And Methods

**PTP1b Assays.** Cell free assays used recombinant, human PTP1b (residues 1-299 expressed and purified from E coli) in 96-well plates containing 200ul DMG buffer (50mM 3,3-dimethyl glutaric acid, I = 0.15M, 0.01% triton, 1 mM EDTA, 1 mM TCEP, pH 7.0) plus 1mM p-nitro phenyl phosphate substrate and various concentrations of inhibitors. Reactions were initiated by the addition of enzyme. Measuring liberation of p-nitro phenyl at OD405 monitored reaction progress, with IC50s calculated based on initial rates. Experiments determining time dependence, enzyme concentration dependence, and effects of detergents on inhibition were as described in the figure legends. Cellular assays of PTP1b activity were as previously described (Cromlish et al., 1999) with minor modifications. Briefly, PTP1b was cloned into the baculovirus transfer vector pBlueBac4.5 (Invitrogen Cat. # V1995-20), and high titre recombinant viral stocks (4.7 x 10^8 pfu/ml) were generated by the manufacturer (Invitrogen) to infect sf21 cells (Invitrogen, Cat. #B821-01) at a multiplicity of infection of 10. Infected cells were harvested by washing and centrifugation using Hank’s solution buffered with 15 mM HEPES, pH 7.4. PTP1b activity was then measured in 200 uL of Hank’s solution containing 5 x 10^4 cells premixed with inhibitors at various concentrations for 30 minutes, followed by addition of 1 mM pNPP (final concentration) to initiate the reaction. Reaction progress was monitored after 15 minutes by measuring OD_{405}.

**Plasmid Construction.** PPARα and PPARγ were cloned from Human Placenta Quick-Clone cDNA (Clontech, Cat. #: 7116-1). PPARγ was cloned by nested PCR, first with the primers 5’-CGAGGACACC GGAGAGGGGC GCCAC-3’ and 5’-GGGAAATGTT GGCAGTGGCT CAGG-3’, followed by the set 5’-TCCGTCGACA AGTCTAGAAC CACCATGACC ATGGTTGACA CAGAG-3’ and 5’-GTGGTGTTGGTG


CCCGGGCTAG TACAAGTCCT TGTA-3’ to allow insertion into the XbaI/SmaI site of pED (Kaufman et al., 1991). PPARα was cloned by PCR into pCR4-TOPO (Invitrogen, Cat. # K4580-01) using the single set of primers 5’-TACTAGTACC ACCATGGTGG ACACGGAAAG CCCAC-3’ and 5’-TACTAGTTCA GTACATGTCC CTGTAGATCT CCT-3’, followed by digestion with SpeI and insertion into the XbaI site of pED. The PPAR response element reporter construct (pGL-3.PPRE) was made using the vector pGL3-promoter (Promega, Cat. # E176A) to allow for regulated expression of luciferase in a background with the SV40 promoter as described by the manufacturer. Three copies of the common PPAR response element (Willson et al., 2000) were inserted as direct repeats using the following dephosphorylated primers annealed together: 5’-CAGGGGACCA GGACAAAGGT CACGTTCGGG AAGGGGACCA GGACAAGGTC ACGTTCGGGA AGGGGACCAG GACAAAGGTC ACGTTCGGGA ACGTTCGGGA C-3’ and 5’-TCGAGTCCCG AACGTGACCT TTGTCCTGGT CCCCTTCCCG AACGTGACCT TTGTCCTGGT CCCCTGGTA C-3’. The annealed oligomers were then cut and ligated into the KpnI and XhoI sites of pGL3-promoter, placing this regulatory sequence 16 bp upstream of the SV40 promoter and 248 bp upstream of the luciferase coding sequence.

**Transcriptional Activation Assays.** CV-1 cells (ATCC, CCL-70) were plated in 12-well plates one day before transfection, grown to 60-80% confluence, and co-transfected with either pED.PPARγ, pED.PPARα, or pED alone along with pGL-3.PPRE (0.1 ug DNA each) using Lipofectamine (Invitrogen, Cat. #18324) according to the manufacturer. Six hours after changing to normal growth medium (DME with 10% FBS), compounds were added and cells were incubated for 40 hours, harvested and assayed for luciferase activity using the Luciferase Assay System (Promega, Cat. # E1501) as instructed.
Adipocyte Differentiation. C3H10T½ Clone 8 cells (ATCC, CCL-226) were grown in 100 mm x 20 mm treated polystyrene cell culture dishes (Corning, Cat. #430167) in DME medium with 10% FBS. One day post confluence, cells were treated with compounds in the presence of 200 nM insulin for two days. Fresh media and test compounds were added (without insulin) for another three days. Accumulation of lipid droplets in cells was determined by staining with the lipophilic dye, Oil Red O (Aldrich, Cat. # 19819-6), by washing once with PBS, fixing with 10% formalin for 10 min, washing once with PBS and once with 60% 2-propanol, and incubating with 0.3% Oil Red O in 60% 2-propanol for 15 min. Cells were then quickly washed once with 60% 2-propanol and once with water prior to visualization and photography.

3T3-L1 Differentiation. 3T3-L1 pre-adipocytes (ATCC, CL-173) were seeded at a density of 2 x 10⁴ cells/ml (2 x 10⁵ cells/dish) and grown to confluence in DME supplemented with 10% calf serum in 100 mm x 20 mm treated polystyrene cell culture dishes (Corning, Cat. #430167). Two days post confluence, media was changed to DME plus 10% FBS, 0.5 mM methylisobutylxanthine, 0.25 µM dexamethasone, and 1 µg/ml insulin for two days followed by incubation in DME 10% FBS plus 1 µg/ml insulin for another 2 days. Finally, cells were incubated in DME with 10% FBS only (no insulin) with fresh media every 2 days until more than 90% were differentiated. Cells were then incubated in serum free DME overnight prior to assay.

Phosphorylation Assay. Following overnight incubation in serum free medium, 3T3-L1 adipocytes were treated with either 10 µM ertiprotfib or 10 µM vanadate for 30 min, plus 10 nM insulin for an additional 10 min. Cells were washed 3 times to remove the insulin, and serum free media with compounds were added back for an additional hour to allow for dephosphorylation of the insulin receptor and IRS-1. Cells were then harvested in
RIPA buffer with protease inhibitors and 1 mM vanadate, vortexed and centrifuged. Supernatants were blotted directly with HRP-conjugated anti-phosphotyrosine clone 4G10 (Upstate Biotechnology, Cat. # 16-105), followed by detection with ECL (Amersham, Cat. # RPN 2106).

**Glucose Uptake.** Differentiated 3T3-L1 adipocytes were incubated in serum-free DME for 2 hours, washed with KPH buffer (136 mM NaCl, 4.7 mM KCl, 1.25 mM CaCl2, 1.25 mM MgSO4, 10 mM Hepes, pH 7.4) and pre-treated with DMSO or ertiprotifib in KPH buffer for 30 minutes. 100 nM insulin (or buffer) was then added for 15 minutes prior to addition of 2-Deoxy-D-[2,6-3H]glucose (Pharmacia, Cat. # TRK672) at 5 µM (1 µCi/mL) for various times. Glucose transport was stopped by three rapid washes in ice cold PBS. Cells were solubilized in 0.1% sodium dodecyl sulfate (SDS) and incorporated radioactivity was quantified by liquid scintillation.

**Animals.** Male Ob/ob mice were purchased from the Jackson Laboratory (Bar Harbor, ME), and acclimated upon arrival prior to study. They were kept on a 12 hour light – 12-hour dark cycle and fed Rodent Diet #5001 (for mice and rats) from Purina Mills (Pharma Serv, Framingham, MA). Male Zucker fa/fa rats were purchased from Charles River Laboratories, Inc. (Wilmington, MA), and were also acclimated to a 12 hour light – 12 hour dark cycle and fed the Rodent Diet #5001. Compounds were dosed orally by gavage in an aqueous suspension of 2% Tween 80 and 0.5% methylcellulose. All experimental work was conducted in accordance with the humane guidelines for ethical and sensitive care of the Institutional Animal Care and Use Committee of the U.S. National Institutes of Health.

**Glucose, Insulin, and Lipid Measurements.** Whole blood (5 uL) was used for glucose readings via tail nick for measurement using the Ascensia Elite XL glucometer and glucose strips (Bayer Corporation, Elkart, IN, Cat. # 9663) by preloading a strip into the meter and touching the end to a small drop of blood on each tail as instructed by the manufacturer. For
insulin measurements, blood was collected by cardiac puncture, centrifuged at 8000 rpm for 10 minutes, and the serum was collected and stored at ~80°C until used. Insulin levels were quantified by ELISA using the Ultra Sensitive Rat Insulin ELISA Kit (Cat. # 90060) from Crystal Chem. Inc. (Downers Grove, IL) as instructed. Briefly, anti-insulin antibody coated microplates were incubated with 100 uL/well of samples and standards at various concentrations in the sample diluent provided for 2 hr at 4°C. Wells were then aspirated, washed 5X with 300 uL washing buffer (provided), and blotted dry. 100 uL of Insulin Enzyme Conjugate was then added to each well, followed by room temperature incubation for 30 minutes, aspiration, 7X washes, and blotting prior to addition of 100 uL/well of Enzyme Substrate Solution. After 40 minutes at room temperature, reactions were stopped with 100 uL/well Enzyme Reaction Stopping solution and absorbance was measured at 450 nm. After subtracting absorbance at 630 nm, insulin concentrations were calculated from the standard curves established in each experiment. Serum triglyceride levels were determined using a kit (Cat. # TR0100) from Sigma (St. Louis, MO) according to the manufacturers instructions by mixing 10 uL of water, glycerol standards, or samples into 0.8 mL of the Free glycerol Reagent (prepared as instructed) followed by incubation for 15 minutes at room temperature and reading the initial absorbance at 540 nm. 0.2 mL of the Triglyceride Reagent was then added per cuvette, followed by mixing and incubation for an additional 15 minutes at room temperature. After recording each final absorbance at 540 nm, triglyceride levels were calculated (subtracting water blanks) as instructed. Serum free fatty acid levels were determined by colorimetry using the FFA kit (Cat. # 994-75409E) from Wako Chemicals (Richmond, VA). This method measures the non-esterified fatty acids present using acyl-CoA synthetase to produce acyl-CoA, which is then oxidized by added acyl-CoA oxidase, producing hydrogen peroxide. In the presence of peroxidase, the generated hydrogen peroxide condenses 3-methyl-N-ethyl-N-(b-hydroxyethyl)-aniline
(MEHA) with 4-aminoantipyrine and the resultant color is measured spectrophotometrically at 550 nm. Sample preparation, assays, and calculations for free fatty acids levels were performed as instructed by the manufacturer.

**Oral Glucose Tolerance Test.** Food was removed at least 12 hours prior to evaluation of glucose disposal. Following a tail vein glucose measurement, animals were allowed to recover prior to receiving an oral bolus of glucose (2 g/kg). Time points were then collected as indicated using tail vein bleeds for glucose levels as above.

**Palmitoyl-CoA Oxidase Assay.** Liver samples were excised from rats, snap frozen in liquid nitrogen, and stored at -80°C until processed. Liver microsomes were prepared first by homogenization in a teflon-glass Potter S mechanical homogenizer with a 5X volume of 50 mM Tris-HCl (pH 7.4) with 0.25 M sucrose, 154 mM potassium chloride and 1 mM EDTA. Microsomes were then purified by differential ultra-centrifugation (Aitio and Vainio, 1976) and the protein content of each microsomal fraction was determined with the Bio-Rad Protein Assay. Palmitoyl-CoA oxidation was measured with modifications of a previously described method (Gray et al., 1983). Briefly, cuvettes contained 50 µM palmitoyl-CoA, 0.25 mM NAD⁺, 0.1 mM coenzyme A, 5 mM dithiothreitol, 1.5 mM KCN, 10 µM FAD, 0.01% (w/v) Triton X-100, 0.15 mg bovine serum albumin, and 0.8 mg liver homogenate in 1 mL of 50 mM Tris-HCl buffer, pH 7.6. Reference cuvettes had no palmitoyl-CoA. The reaction was monitored every 30 seconds for 30 minutes at 340 nm at 37°C in a Hitachi U-3010 Spectrophotometer. Activity was quantified as nanomoles of NADH formed per minute per milligram of homogenate protein using 6.22 cm⁻¹ mM⁻¹ as an extinction coefficient. All reagents were purchased from Sigma.
Results

Ertiprotfib and a close analog, Compound 3 (see Figure 1 for structures), were originally developed as *in vitro* inhibitors of PTP1b capable of improving insulin sensitivity in mice. Table 1 shows an example of the results following 4 days of treatment with ertiprotfib in the leptin deficient *ob/ob* mouse model. Here, 11 week old male *ob/ob* mice were placed into two similar groups by weight and fasting blood glucose, and then dosed with either vehicle (2% Tween 80, 0.5% methyl cellulose) or ertiprotfib at 25 mg/kg/day for 4 days. Following treatment, mice treated with ertiprotfib had their fasting glucose levels returned to the normal range in the context of markedly reduced insulin levels. Thus, ertiprotfib appeared to increase insulin sensitivity in these mice following short-term treatment. Notably, serum triglyceride levels were also markedly reduced in these treated animals (see below).

To further test the ability of ertiprotfib to increase insulin sensitivity, male Zucker *fa/fa* rats were dosed with ertiprotfib or Compound 3 at either 25 mg/kg/day or 5 mg/kg/day for 14 days. Fasting blood glucose levels were measured and an oral glucose tolerance test (OGTT) was performed on day 10 of dosing, while insulin, triglyceride, and free fatty acid levels were measured at the end of the 14-day dosing. As was seen with treatment of *ob/ob* mice, both compounds appeared to significantly improve glucose metabolism in rats (Table 2 and Figure 2). At 25 mg/kg/day these compounds decreased both fasting blood glucose and insulin levels when compared to vehicle treated rats (Table 2). Furthermore, both ertiprotfib and Compound 3 increased glucose disposal following an oral challenge (Figure 2). Compound 3 appeared to work slightly better at this dose, consistent with its two-fold greater exposure following oral dosing (data not shown), but neither compound was efficacious at the 5 mg/kg/day dose. Importantly, lipid levels were
also reduced in treated animals (Table 2). Both triglyceride and free fatty acid levels were substantially reduced in rats treated with 25 mg/kg/day of either compound. Again, Compound 3 may be slightly more potent, with free fatty acids levels reduced even at the 5 mg/kg/day dose of this compound. To summarize, both ertiprotapib and Compound 3 appear to be robust agents in improving glucose utilization in fa/fa rats, while also decreasing lipid levels in these animals.

Decreased lipid levels (especially free fatty acids) may be unexpected for a pure PTP1b inhibitor (Klaman et al., 2000). More telling, as mentioned above, rats treated with supra-pharmacologic doses of ertiprotapib showed signs of PPAR family activation (peroxisome proliferation, adipose deposition in bone marrow) (Peterson et al., 2004). Because engagement of PPAR family members could contribute to the efficacy seen here, we re-examined the actions of ertiprotapib in vitro to gain a more complete understanding of its mechanism(s) of action.

Here, we see that ertiprotapib does not behave as a simple, competitive inhibitor of PTP1b. Inhibition follows a time dependent, irreversible mechanism that displays non-classical kinetics (Figure 3a and Table 3). Most telling, this inhibition is dependent on the concentration of enzyme used in each assay, a hallmark of complex inhibition (Table 3). In addition, IC50s for this class of compounds in cell-free assays of PTP1b enzymatic activity vary depending on each assay's components and their interactions with these non-polar compounds (data not shown). For example, adding small amounts of the nonionic detergent, triton, shifts the inhibition curve with ertiprotapib (Table 3). Similar results have been found for compounds related to this series (Johnson et al., 2002; Burkey et al., 2002). Thus, PTP1b inhibition by ertiprotapib must be understood in the context of compounds with similar, complex behavior (McGovern et al., 2002; Doman et al., 2002).
To define the concentrations required to inhibit PTP1b in the cellular milieu, we first used a simplified cellular assay expressing recombinant PTP1b in the baculovirus system in sf21 insect cells and measuring turnover of pNPP as previously reported (Cromlish et al., 1999). In this assay (Figure 3b), both vanadate and the known, reversible PTP1b inhibitor, Compound #33 (see Figure 1 for structure from Taing et al., 1999), give inhibition constants of 0.1 uM and 4.5 uM respectively, consistent with literature values (Cromlish et al., 1999; Taing et al., 1999; Huyer et al., 1997). Ertiprotafib, on the other hand, inhibits with an IC50 greater than 20 uM (Figure 3b). Similar results were found for Compound 3, with an IC50 greater than 20 uM (not shown). As in cell free assays, inhibition by ertiprotafib (but not the control compounds vanadate and Compound #33) in this cellular assay varied with assay components and with time, with greater inhibition seen at lower enzyme levels and later time points (not shown).

Next, potential effects of ertiprotafib on PTP1b regulation of insulin signaling were investigated in 3T3-L1 cells differentiated into adipocytes by standard procedures (Frost and Lane, 1985). Cells were pre-incubated with inhibitor for 30 minutes, pulsed with insulin for 10 minutes, washed and compounds were added back for another hour prior to harvest, lysis, electrophoresis and blotting with anti-phosphotyrosine antibodies. This time after insulin withdrawal allows measurement of the rate of dephosphorylation of the insulin receptor and proximal signaling molecules in the presence of inhibitors. Although no significant difference in phosphorylation of the insulin receptor was seen with either vanadate or ertiprotafib, both treatments did give about a two-fold increase in tyrosine phosphorylation of the downstream molecule IRS-1 (Figure 4a). The short incubation period makes it likely that this effect is through phosphatase inhibition, presumably PTP1b, although at these concentrations effects on other phosphatases cannot be ruled out. This increase in IRS-1 phosphorylation did not translate into increased glucose uptake by these cells, though. As
seen in Figure 4b, no increase in glucose uptake by the differentiated 3T3-L1 cells was seen with ertiprotapfrib either alone or with insulin. Similar results were seen with cultured myocytes (not shown). Taken together, these assays of phosphatase inhibition and insulin signaling indicate that although ertiprotapfrib inhibits PTP1b in cells, this inhibition occurs at relatively high concentrations (under the specified assay conditions) and may not provide a full explanation of its \textit{in vivo} efficacy.

To evaluate whether agonism of PPAR family members might play a role in the pharmacology of ertiprotapfrib, a reporter system was employed using luciferase as the readout. Three copies of the DNA for the consensus PPAR response element (PPRE) were inserted upstream of the SV40 promoter and luciferase gene (reporter plasmid pGL3). This was co-transfected into CV-1 cells along with expression plasmids for either PPAR\(\alpha\) or PPAR\(\gamma\). Test compounds were then added at various concentrations for 40 hrs (to allow expression of each PPAR and induced transcription of luciferase) prior to cell lysis and quantification of luciferase activity. As seen in Figure 5, ertiprotapfrib appears to activate transcription through both PPAR\(\alpha\) and PPAR\(\gamma\) at concentrations similar to known agonists. Here, ciprofibrate gave dose-dependent stimulation of PPAR\(\alpha\) driven transcription of luciferase (EC50 of 0.6 +/- 0.2 uM). Ertiprotapfrib also appeared to drive PPAR\(\alpha\) dependent transcription to the same extent, with an EC50 of 1.4 +/- 0.5 uM. In Figure 5b, one sees that ertiprotapfrib also activates transcription through PPAR\(\gamma\) (EC50 of 1.1 +/- 0.4 uM) although the magnitude of the response was less than the known PPAR\(\gamma\) agonist, troglitazone (EC50 of 1.7 +/- 0.1 uM), indicating partial agonism of this receptor by ertiprotapfrib. Ertiprotapfrib activation of each PPAR appears to be direct. Figure 6 shows the results of experiments using ertiprotapfrib to compete for binding of fluorescent fatty acids to each PPAR. Here, we see competitive displacement of fatty acid binding to PPAR\(\alpha\) with an IC50 of 0.5 uM.
(Figure 6a) and displacement of fatty acid binding to PPARγ with an IC50 of 0.6 uM (Figure 6b).

The transactivation of PPARγ by ertiprotafib also results in relevant responses in cells, as measured by adipocyte differentiation of the murine fibroblast line C3H/10T 1/2 (Figure 7). This assay was used in identification of PPAR ligands and has been clearly shown to reflect induction of the adipocyte phenotype in a PPARγ dependent manor (Forman et al., 1995). In these assays, fibroblasts are treated with compounds and insulin for a total of 4-6 days, washed, fixed, and stained with oil red to visualize the fat droplets that form as a result of adipogenesis. In Figure 7 one sees the comparison between control cells treated with DMSO alone (A) and cells treated with the known PPARγ agonist rosiglitazone (B), or the test compounds ertiprotafib (C) and Compound 3 (D). One can clearly see the increase in cells staining positive for fat droplets with oil red when treated with rosiglitazone (Figure 7, B). Ertiprotafib and Compound 3 were evaluated in these same cells and also appeared to drive adipogenesis (Figure 7, C and D). Therefore, not only does ertiprotafib transactivate PPARγ in the reporter gene systems, it also serves as bona fide inducer of adipogenesis in fibroblast cells. In fact, when compared with rosiglitazone, a known PPARγ agonist, both ertiprotafib and its analog improve glycemic excursion to a similar extent following an oral glucose tolerance test in fa/fa rats (Figure 8).

Lastly, one sees evidence for the relevance of PPARα engagement in vivo by ertiprotafib, through induction of a PPARα responsive enzyme in treated animals (Figure 9). Here, CN⁻ insensitive Palmitoyl-CoA oxidase activity was measured in the isolated microsomes of liver from animals treated with either ertiprotafib or rosiglitazone, versus vehicle treated and lean controls. One sees no difference in activity in fa/fa rats treated with either vehicle or rosiglitazone (even compared to lean controls). Ertiprotafib treatment,
However, significantly increased oxidase activity (Figure 9). Thus, one sees evidence of PPARα engagement by ertiprotafib under conditions where efficacy is seen.

**Discussion**

Ertiprotafib was initially targeted as a PTP1b inhibitor for the treatment of type 2 diabetes and progressed as far as phase II clinical trials prior to discontinuation of its development. The present study sought to clarify its mechanism(s) of action to aid in interpretation of these results. In this study, PTP1b inhibition by ertiprotafib was shown to follow non-classical kinetics, with results dependent on the time of incubation as well as assay components such as enzyme concentration. Recently, a common mechanism has been described for many of the poorly behaved inhibitors that result from screening efforts (McGovern et al., 2002; Doman et al., 2002). Compounds of this type form higher order aggregates that have been visualized by electron microscopy. Their inhibitory properties are dependent upon this aggregation, resulting in non-classical kinetics. Two signatures of such inhibition are changes in IC50s with either a change in enzyme concentration or addition of a small amount of detergent (McGovern et al., 2002; Brian Shoichet, unpublished). Both phenomena were seen in the characterization of ertiprotafib. This demonstration of poorly behaved kinetics confounds estimates of potency vs. PTP1b *in vivo*. Ertiprotafib did appear here to inhibit PTP1b in cellular assays (including delaying de-phosphorylation of IRS-1 in adipocytes) albeit at relatively high concentrations. Ertiprotafib treatment of either fat or muscle cells did not translate into a short term increase in glucose uptake, although these assays may not be sensitive enough to see a partial effect of PTP1b inhibition. Thus, these data indicated that although PTP1b inhibition likely plays some role in the pharmacology of
ertiprotafib, potent inhibition of PTP1b seems an unlikely explanation for its complete pharmacological profile.

The data presented here indicate that (in addition to inhibiting PTP1b) ertiprotafib is at least a dual PPAR\(\alpha\) and PPAR\(\gamma\) agonist with EC50s for transactivation of ~1 \(\mu\)M. Such activities readily explain the observations with supra-pharmacologic doses of these compounds (see above) while also contributing to the pharmacology seen at efficacious doses. The efficacy results presented show that ertiprotafib and its analog, Compound 3, lowered plasma glucose levels, reduced insulinemia, lowered serum triglycerides, and lowered free fatty acid levels in rodent models of type 2 diabetes. Importantly, similar results were seen when these same animal models were used in the evaluation of TZD’s (Spiegelman, 1998). Although a PTP1b inhibitor could be expected to decrease glucose, insulin, and triglyceride levels based upon the phenotype of the knockout mice, free fatty acid levels were not different in these animals (Klaman et al., 2000). PPAR\(\alpha\) ligands, however, are able to lower both serum triglycerides and free fatty acids in rodents (Staels et al., 1998; Chou et al., 2002), and are able to improve insulin sensitivity (Guerre-Millo et al., 2000). Thus, given the increase in acyl-CoA oxidase activity in the livers of treated animals, it seems likely that PPAR\(\alpha\) agonism (in addition to PPAR\(\gamma\) agonism and PTP1b inhibition) may contribute to the drop in fatty acid levels seen with ertiprotafib treatment, along with a portion of the glucose and insulin lowering. In fact, the livers of Sprague-Dawley rats treated with ertiprotafib showed widespread up-regulation of genes involved in lipid metabolism, peroxisome proliferation, and fatty acid \(\beta\)-oxidation (Peterson et al., 2004). These animals also exhibited increased liver weights and hepatocellular hypertrophy, along with decreased cholesterol and triglyceride levels. Furthermore, many of the genes with increased liver expression following ertiprotafib treatment are known to be
PPARα-regulated, including acyl-CoA oxidase, PEX11α, and CYP4A1 (Peterson et al., 2004). These animals also showed induction of the PPARγ-regulated gene CD36 in ovaries as well as multiple PPARγ-dependent genes in fat (Peterson et al., 2004; and unpublished). Although these studies were performed at doses of ertiprotafib higher than those needed for efficacy, the results are consistent with the data presented here and indicate that PPAR agonism likely contributes to the pharmacology seen with ertiprotafib. In fact, preliminary experiments indicate that ertiprotafib is able to transactivate gene expression through PPARδ as well, with an EC50 of 5 μM (CR Elcombe, unpublished). Since agonism of PPARδ can be expected to mediate similar effects in vivo to those shown here (Wang et al., 2003), this nuclear receptor may also contribute to the pharmacology observed with ertiprotafib. What portion each of the targets discussed here (PTP1b, PPARα, PPARγ, PPARδ) may play in the pharmacology of ertiprotafib, as well as potential contributions from other targets (e.g., RXR), requires further study.

In conclusion, results from both in vitro and in vivo experiments indicate that the improvements in peripheral insulin sensitivity (normalizing plasma glucose while reducing insulin levels) and reductions in hyper-lipidemia following treatment with ertiprotafib are likely due to a combination of mechanisms. These data should inform interpretations from results of clinical trials with ertiprotafib and aid in the search for improved insulin sensitizers.
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Legends for Figures

**Fig. 1.** Structures of ertiprotafib and Compound 3.

**Fig. 2.** Ertiprotafib and Compound 3 increase glucose disposal in Zucker *fa/fa* rats. Male Zucker *fa/fa* rats were dosed orally for 10 days (see Methods) with vehicle, ertiprotafib, or Compound 3 at 25 mpk or 5 mpk per day as indicated. Following a 12-hour fast, animals received an oral bolus of glucose (2 g/kg) and blood glucose was measured at the indicated times by tail bleeds.

**Fig. 3.** A. Ertiprotafib inhibition is time dependent. pNPP cleavage by PTP1b was monitored over time in the presence of the indicated concentrations of ertiprotafib as described in the Methods. B. Inhibition of PTP1b in sf21 cells. PTP1b cleavage of pNPP was measured using sf21 cells (see Methods) following a 30-minute pre-incubation with the indicated concentrations of vanadate (open squares), Compounds #33 (open triangles), or ertiprotafib (closed squares).

**Fig. 4.** Effect of ertiprotafib signaling in 3T3-L1 adipocytes. A. IR and IRS-1 dephosphorylation following insulin withdrawal was measured in 3T3-L1 adipocytes treated with DMSO (lane 1), 10 uM vanadate (lane 2), or 10 uM ertiprotafib (lane 3) prior to blotting with anti-pTyr antibodies (see Methods). Identities of each band were confirmed by stripping and blotting with IR-β and IRS-1 specific antibodies (not shown). B. Glucose uptake by 3T3-L1 adipocytes was measured after incubation with or without insulin as indicated either alone (open bars) or with 20 uM ertiprotafib (filled bars) as described in the Methods.
Fig. 5. Ertiprotafib activates transcription through PPARα and PPARγ. A. CV-1 cells were transfected with the luciferase reporter construct (pGL-3-PPRE) and a PPARα expression construct (pED.PPARα) prior to treatment with cipprofibrate (filled squares) or ertiprotafib (open squares) and measurement of induced luciferase activity as described in the Methods. B. CV-1 cells were transfected with the luciferase reporter construct (pGL-3-PPRE) and a PPARγ expression construct (pED.PPARγ) prior to treatment with troglitazone (filled diamonds) or ertiprotafib (open squares) and measurement of induced luciferase activity as described in the Methods. Luciferase activity is expressed in arbitrary units following subtraction of background luminescence resulting from cells transfected with empty plasmids.

Fig. 6. Ertiprotafib competes for binding of fatty acids to PPARα and PPARγ. A. The recombinant PPARα ligand binding domain was expressed in E. Coli as a poly-his tagged fusion and purified by nickel affinity chromatography for use in a competitive displacement assay with the fluorescent fatty acid, 12-anthracene oleic acid as previously described (Palmer and Wolf, 1998; Causevic et al., 1999). Results are expressed as the percentage of 12-anthracene oleic acid bound (following subtraction of background fluorescence from protein or fatty acid alone) in the presence of the indicated concentrations of ertiprotafib, compared to binding in the absence of ertiprotafib. B. Ertiprotafib was assayed for its ability to bind PPARγ as in (A) but with cis-parinaric acid as the reporter ligand, again as described (Palmer and Wolf, 1998; Causevic et al., 1999).

Fig. 7. Ertiprotafib and its analog induce adipocyte differentiation. C3H10T½ cells were differentiated into adipocytes (see Methods) in the presence of either (A) DMSO, (B) 50 nM
rosiglitazone, (C) 10 uM ertiprotifib, or (D) 10 uM Compound #3 prior to staining with Oil Red O to visualize fat droplets.

**Fig. 8.** Ertiprotifib and rosiglitazone increase glucose disposal to comparable levels. Male Zucker *fa/fa* rats were dosed for 14 days with either vehicle (2% Tween 80, 0.5% methyl cellulose), 3 mpk rosiglitazone, or 25 mpk ertiprotifib as described in the Methods. Following a 12 hour fast, treated animals (as well as untreated lean controls) received an oral bolus of glucose (2 g/kg) prior to measurement of blood glucose levels by tail bleeds (see Methods).

**Fig. 9.** Ertiprotifib induces fatty acid oxidation in the livers of treated animals. Livers from the Zucker lean and *fa/fa* rats (see Figure 7) treated 14 days of dosing with either 3 mpk rosiglitazone, 25 mpk ertiprotifib, or vehicle were assayed for NADH production as described in the Methods. For each group n = 4. *P*<0.005 by ANOVA compared to vehicle treated animals.

**Tables and Figures (see following pages)**
Table 1: Ertiportafib lowers glucose, insulin, and triglycerides in *ob/ob* mice.

<table>
<thead>
<tr>
<th></th>
<th>Body Weight before (g)</th>
<th>Body Weight after (g)</th>
<th>FBG before mg/dL</th>
<th>FBG after mg/dL</th>
<th>Insulin after ng/ml</th>
<th>TRIG after mg/dL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>48.1 +/- 0.8</td>
<td>48.0 +/- 1.0</td>
<td>101 +/- 4</td>
<td>102 +/- 10</td>
<td>8.4 +/- 0.7</td>
<td>42.7 +/- 1.5</td>
</tr>
<tr>
<td>Erti</td>
<td>48.1 +/- 0.8</td>
<td>49.5 +/- 0.8</td>
<td>102 +/- 4</td>
<td>76 +/- 2</td>
<td>2.5 +/- 0.5</td>
<td>32.9 +/- 2.5</td>
</tr>
</tbody>
</table>

* p < 0.05 compared to vehicle  
* p < 0.01 compared to vehicle  
* p < 0.001 compared to vehicle  
* p < 0.0001 compared to before treatment
**Table 2:** Ertiportafib and Compound 3 lower glucose, insulin, triglycerides, and free fatty acids in fa/fa rats.

<table>
<thead>
<tr>
<th>rats</th>
<th>treatment</th>
<th>Dose</th>
<th>FBG mg/dL</th>
<th>Insulin ng/ml</th>
<th>TRIG mg/dL</th>
<th>FFA mol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>lean</td>
<td>- - -</td>
<td>- - -</td>
<td>68 +/- 5</td>
<td>3.7 +/- 0.3</td>
<td>118 +/- 11</td>
<td>640 +/- 50</td>
</tr>
<tr>
<td>fa/fa</td>
<td>vehicle</td>
<td>- - -</td>
<td>115 +/- 18</td>
<td>15.8 +/- 1.8</td>
<td>1060 +/- 190</td>
<td>1620 +/- 160</td>
</tr>
<tr>
<td>fa/fa</td>
<td>ertiprotafib</td>
<td>25 mg/kg</td>
<td>78 +/- 5*</td>
<td>9.8 +/- 0.7*</td>
<td>260 +/- 30*</td>
<td>560 +/- 40*</td>
</tr>
<tr>
<td>fa/fa</td>
<td>ertiprotafib</td>
<td>5 mg/kg</td>
<td>99 +/- 4</td>
<td>13.1 +/- 1.4</td>
<td>770 +/- 230</td>
<td>1160 +/- 210</td>
</tr>
<tr>
<td>fa/fa</td>
<td>Cmpd #3</td>
<td>25 mg/kg</td>
<td>77 +/- 8*</td>
<td>7.3 +/- 0.6*</td>
<td>147 +/- 18*</td>
<td>450 +/- 40*</td>
</tr>
<tr>
<td>fa/fa</td>
<td>Cmpd #3</td>
<td>5 mg/kg</td>
<td>92 +/- 3</td>
<td>13.6 +/- 1.1</td>
<td>550 +/- 70</td>
<td>870 +/- 40*</td>
</tr>
</tbody>
</table>

* p < 0.05 compared to vehicle
Table 3: Inhibition by Ertiprotafib: Effects of [E] and Detergent

<table>
<thead>
<tr>
<th>Conc PTP1b$^a$</th>
<th>no triton</th>
<th>0.01 % triton</th>
</tr>
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<tbody>
<tr>
<td>10 nM</td>
<td>1.6 µM</td>
<td>16 µM</td>
</tr>
<tr>
<td>100 nM</td>
<td>9 µM</td>
<td>29 µM</td>
</tr>
</tbody>
</table>

$^a$IC50s were determined for inhibition of pNPP turnover as in the Methods at two different enzyme concentrations in the presence or absence of added triton.
Figure 1
Figure 2
Figure 3

A

OD 405 vs. time (seconds)

- Line 1: µM erti 0
- Line 2: µM erti 6.3
- Line 3: µM erti 12.5
- Line 4: µM erti 25.0

B

OD 405 vs. µM inhibitor

- □: µM erti 0
- ■: µM erti 6.3
- ▲: µM erti 12.5
- ■: µM erti 25.0
Figure 4
Figure 5
Figure 6
Figure 7
Figure 8
Figure 9

NADH production
(nmol/min/mg)

Lean
Vehicle
Rosii
Erri

*