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Molecular Characterization of Novel and Selective Peroxisome Proliferator-Activated Receptor α Agonists with Robust Hypolipidemic Activity in Vivo[S]

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ABSTRACT

The nuclear receptor peroxisome proliferator-activated receptor α (PPARα) is recognized as the primary target of the fibrate class of hypolipidemic drugs and mediates lipid lowering in part by activating a transcriptional cascade that induces genes involved in the catabolism of lipids. We report here the characterization of three novel PPARα agonists with therapeutic potential for treating dyslipidemia. These structurally related compounds display potent and selective binding to human PPARα and support robust recruitment of coactivator peptides in vitro. These compounds markedly potentiate chimeric transcription systems in cell-based assays and strikingly lower serum triglycerides in vivo. The transcription networks induced by these selective PPARα agonists were assessed by transcriptional profiling of mouse liver after short- and long-term treatment. The induction of several known PPARα target genes involved with fatty acid metabolism were observed, reflecting the expected pharmacology associated with PPARα activation. We also noted the down-regulation of a number of genes related to immune cell function, the acute phase response, and glucose metabolism, suggesting that these compounds may have anti-inflammatory action in the mammalian liver. Whereas these compounds are efficacious in acute preclinical models, extended safety studies and further clinical testing will be required before the full therapeutic promise of a selective PPARα agonist is realized.

Coronary heart disease (CHD) is the single leading cause of death in the United States, representing one of every five fatalities during 2004 (Rosamond et al., 2007). Elevated serum triglycerides are an independent risk factor for CHD irrespective of HDL cholesterol (Hokanson and Austin, 1996). The fibric acid derivatives (fibrates) represent one therapeutic option for the treatment of dyslipidemia in that they display robust triglyceride lowering with modest HDL elevation. Significant effort has been put forth in the development of pharmacologic agents that demonstrate clear superiority over the first generation fibrates for the treatment of dyslipidemia and CHD.

The fibrates can directly bind and activate a subclass of the nuclear receptor superfamily know as the peroxisome proliferator-activated receptors (PPARs). The PPARs are ligand-dependent transcription factors that are classified as three subtypes known as PPARα, PPARβ (δ), and PPARγ. The PPARs are recognized as major regulators of systemic energy metabolism, in part by controlling the expression of numerous genes involved in lipid and glucose metabolism (Desvergne and Wahli, 1999; Huss and Kelly, 2004). More recently, an important and emerging role for the PPARs

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ABBREVIATIONS: CHD, coronary heart disease; HDL, high density lipoprotein; PPAR, peroxisome proliferator-activated receptor; PGC-1α, peroxisome proliferator-activated receptor γ coactivator-1α; CP-865520, (*S*)-2-methyl-2-(3-(1-(2-(4-(trifluoromethoxy)phenyl)acetyl)piperidin-3-yl)phenoxy)propanoic acid sodium salt; CP-775146, (*S*)-2-(3-(1-(2-(4-isopropylphenyl)acetyl)piperidin-3-yl)phenoxy)-2-methylpropanoic acid sodium salt; CP-868388, (*S*)-2-(3-(1-((4-isopropylbenzyloxy)carbonyl)piperidin-3-yl)phenoxy)-2-methylpropanoic acid sodium salt; '520, CP-865520; '146, CP-775146; '388, CP-868388; LBD, ligand binding domain; GST, glutathione transferase; RT, room temperature; ANOVA, analysis of variance; β-gal, β-galactosidase; DMSO, dimethyl sulfoxide; PCR, polymerase chain reaction; Wy14,643; APR, acute phase response; CEBP, CCAAT/enhancer-binding protein; g-6-P, glucose 6-phosphate; SRC-1, steroid receptor coactivator-1.

relating to both the etiology and treatment of inflammatory disease such as atherosclerosis has been appreciated (Devchand et al., 2004; Li et al., 2004). Small-molecule agonists have been developed and approved for therapeutic use. Fibrates represent weak and relatively nonselective agonists of PPARα, whereas the thiazolidinediones exhibit greater potency and selectivity for PPARγ. They are currently used to treat dyslipidemia and diabetes, respectively.

To take advantage of the therapeutic potential of both fibrates and thiazolidinediones, researchers in the field have pursued novel agents with vastly improved potency and varying degrees of selectivity for the PPARs (Evans et al., 2004). Unfortunately, this strategy has not proven successful in the clinic. Numerous agents have failed at multiple stages of clinical testing, often as a result of muscle- and/or cardiac-related toxicities, including rhabdomyolysis and congestive heart failure and as well as carcinogenicity (Rubenstrunk et al., 2007). In addition, concomitant activation of multiple PPAR subtypes within the same or multiple target tissues may increase the probability of deleterious side effects (Rubenstrunk et al., 2007). We have therefore hypothesized that a highly selective PPARα agonist with improved potency should enhance the lipid-lowering efficacy obtained with a fibrate. In addition, such a compound may provide greater anti-inflammatory potential and avoid the untoward toxicities associated with nonselective PPAR activation. To this end, we report the chemical structure of a novel and selective PPARα chemotype and the characterization of its molecular mechanism of action using a combination of in vitro, in vivo, and microarray techniques. These studies demonstrate the therapeutic promise of a selective PPARα agonist.

Materials and Methods

Materials. The compounds CP-865520, CP-775146, and CP-868388 were synthesized at Pfizer, Inc. (Groton, CT). In all experiments, analytical grade reagents were used.

Biochemical Assays. Recombinant GST:PPAR LBD proteins (human) were prepared using standard techniques whereas ^{3}H radioligands and piperidine agonists were synthesized at Pfizer Inc. Rabbit α-GST antibody (Invitrogen, Carlsbad, CA), YSi α-rabbit scintillation proximity assay beads (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK), and GST:PPAR were added to phosphate-buffered saline and mixed for 1 h at RT. Radioligands and agonists were added to the bead cocktail, sealed in a 96-well plate, and shaken for 1 h at RT. Beads were allowed to settle and read on a Wallac Microbeta plate reader (PerkinElmer Life and Analytical Sciences, Waltham, MA). Samples were assayed in duplicate during two or more independent experiments, and statistical significance was determined using the Student's *t* test. Fluorescence resonance energy transfer was performed by mixing 20 nM GST:PPARα LBD, 40 nM biotin-labeled coactivator peptide (SRC-1, Biotin-SLTARHKILHRLLQEGSPSDI, PGC-1α, Biotin-EAEEPSLLKKLLLAPANTQ) 2 nM europium labeled α-GST antibody (Perkin Elmer), 40 nM streptavidin Surelight-APC (PerkinElmer Life and Analytical Sciences) in buffer (50 mM Tris, pH 8, 50 mM KCL, 1 mM EDTA, and 0.1 mg/ml bovine serum albumin) and incubated at 4°C with agonist for 1 h and read on a Victor2 plate reader (PerkinElmer Life and Analytical Sciences). Bound versus unbound ratios (665 nm/615 nm) were determined, results were plotted as -fold over background (0 μM ligand 665/615 ratio), and statistical significance was determined using ANOVA with a Bonferroni correction. Samples were assayed in triplicate during three or more independent experiments.

Cell Culture, Transfection, and Reporter Assays. HepG2 cells were transfected under standard conditions with 50 ng 5× Gal4 response element-luciferase reporter, 40 ng of Gal4:NHR-LBD (human PPARα amino acids, 187–468; human PPARγ, 196–477), and 25 ng of cytomegalovirus β-gal using FuGENE 6 (Roche, Indianapolis, IN). Transfected cells (n = 3/condition) were treated with compounds for 18 to 24 h, lysed, and luciferase and β-gal assays performed (Promega, Madison, WI) using a luminometer (Dynex Technologies, Chantilly, VA) and a plate reader (Molecular Devices, Sunnyvale, CA). Luciferase values were corrected for transfection efficiency using β-gal and are shown as -fold induction versus Gal4:PPARα treated with DMSO. Statistical significance was determined using ANOVA with a Bonferroni correction with samples assayed in triplicate during three or more independent experiments.

In Vivo Pharmacology. Drug exposure of each compound was compared in fasted rats (n = 2) orally gavaged with a 3.0 mg/kg (2 ml/kg) dose. Rats were fed 4 h after dosing. Blood was extracted through the jugular cannula, and drug concentrations in plasma determined using liquid chromatography/mass spectrometry. Data shown represent mean values. Male *B6/CBF1J* mice (n = 5) were maintained on a chow diet and were orally gavaged with a single dose of either vehicle (water) or agonist for 2 days. Mice were sacrificed on the third day (72 h after initiation of study), and plasma was prepared using standard techniques. Plasma triglycerides were quantified using the L-type triglyceride-H kit from Wako Chemicals (Richmond, VA). Statistical significance was determined using ANOVA with a Bonferroni correction. The study protocols were approved by the Institutional Animal Care and Use Committee and all animals received humane treatment according to the criteria stated by the National Academy of Sciences National Research Council (NRC) publication 86-23, 1985.

Expression Profiling, Real-Time PCR, and Pathway Mapping. Wild-type or PPARα-null male *C57BL/6* mice (n = 4) were treated with the piperidine agonists for 24 h or 5 days at 1 mg/kg/day. Total liver RNA was isolated using an RNeasy mini kit (QIAGEN, Valencia, CA) and cDNA, and subsequent biotinylated cRNA was prepared according to published Affymetrix (Santa Clara, CA) protocols. Labeled cRNA (10 μg) was hybridized onto the Affymetrix MG_U74Av2 GeneChips overnight, washed, stained with a SAPE solution, and images captured using the GeneChip scanner (Agilent Technologies, Santa Clara, CA). Absolute intensity values were generated using the MAS5 algorithm then scaled to 300 for plate-to-plate comparisons. For the treated versus vehicle animals ratios, -fold change values were calculated, with intensity values less than 50 being adjusted to 50. A -fold change of ± 2.5-fold and a corresponding p-value of ≤ 0.05 (Student's t test) occurring in at least 1 compound was applied for determination of differential expression (compound versus vehicle). For the CP-775146 cohort at 5 days (n = 2), -fold change alone was used to identify differentially regulated messages. Selection of differential gene expression for the treated PPARα-null animals was calculated as those probe sets where the -fold-change for any two of three agonist exposed groups was ±1.05-fold with a p value of ≤ 0.05 (Student's t test). Data visualization and categorization was performed using the Spotfire Decision Site software package and hierarchical clustering applying the Pearson Correlation similarity measurement to the log10 ratio of the 538 differentially expressed genes was done using GeneSpring GX. TaqMan (quantitative real-time PCR) was performed with an aliquot of the ds cDNA from the microarray study using 2× TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA) under standard assay conditions of 40 cycles with commercially available probes (Applied Biosystems) with a Thermocycler (PE 5700; Applied Biosystems). $\Delta\Delta C_t$ comparisons were made using GAPDH as the endogenous comparator and -fold changes were calculated against the vehicle control samples. Molecular network relationships for the 538 differentially expressed genes were determined with the Ingenuity Pathway Analysis tool (Ingenuity Systems, Redwood City, CA). Inclusion criteria for this analysis included any message with a ±1.5-fold change that was common across all three compounds at either 24 h or 5 days. All tables containing gene expression data were

created with this tool and display the human gene name and description coupled to the murine Affymetrix identifier used in the microarray study. The dataset has been deposited at NCBI Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) with accession number GSE12147.

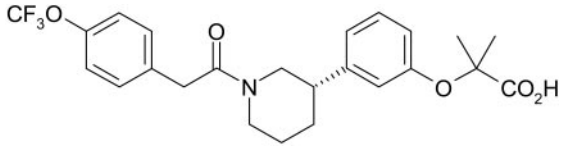
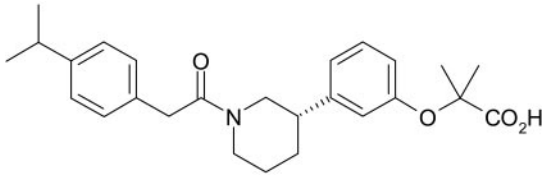
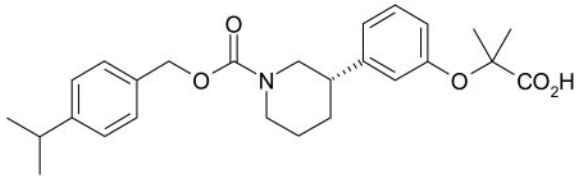
Results

Identification of Novel and Potent Synthetic Agonists for PPARα. The fibrates are weak and relatively nonselective agonists of PPARα that display triglyceride lowering and modest HDL elevation in preclinical animal models and human patients. Efforts in our laboratories have focused on improvements to both potency and selectivity of the historic fibrates and have lead to the discovery of the novel piperidine-based series of PPARα agonists, the structures of which are displayed in Table 1. To characterize the binding potency and selectivity of the PPAR ligands, we used a scintillation proximity assay for each human PPAR subtype (α, β, and γ) in which a reference ligand was bound to the purified LBD and then displaced by the addition of test compound. First-generation fibrates, including ciprofibrate, gemfibrozil, and fenofibrate, demonstrate very weak PPARα binding in vitro with spanning 58 to >200 μM. (Mukherjee et al., 2002). As observed in Table 1, the piperidine agonists bound PPARα very strongly with K_i values of 74 nM for CP-865520 ('520), 24.5 nM for CP-775146 ('146), and 10.8 nM for CP-868388 ('388). The apparent increasing affinity within the piperidine class was statistically significant ($p < 0.05$). These compounds had no observable affinity for PPARβ (selectivity ratios, 140–925-fold) and little or no affinity for PPARγ (selectivity ratios, 140–400-fold). The apparent weak (3.47 μM) K_i of '388 for PPARγ was not found to be statistically significant relative to the other compounds.

Improved Recruitment of Cofactors by Piperidine Agonists. Numerous structural and biochemical studies have demonstrated that the binding of nuclear receptor agonists promote conformational changes within the LBD that enable cofactor recruitment (Nolte et al., 1998; Xu et al., 2001). We therefore characterized the ability of recombinant human PPARα LBD to recruit coactivator peptides in a ligand-dependent manner as a surrogate for full-length cofactor binding. As shown in Fig. 1A, the weak PPARα agonist fenofibrate was able to recruit the SRC-1 peptide in a dose-dependent manner with an induction window of nearly 4-fold. The piperidine agonists were also found to be robust recruiters of SRC-1, with a similar or slightly better induction window. The improved potency of the piperidine agonists was reflected by the left-shift of the dose-response curve by nearly 22-fold versus fenofibrate and is highly significant ($p < 0.00001$). The SRC-1 EC_{50} values for '520, '146, and '388 (8.5, 6.3, and 4.7 nM, respectively) were in the same rank order as observed in the scintillation proximity assay but were not statistically different from one another. Similar results were obtained with an additional coactivator peptide taken from PGC-1α (Fig. 1B). All compounds displayed robust and dose-dependent recruitment of PGC-1α peptide whereas the piperidine agonists demonstrated improved potency versus fenofibrate.

Piperidine Agonists Demonstrate Robust and Selective Transcriptional Activation of PPARα in Cell Culture. The transactivation potential of the piperidine agonists were assessed in HepG2 cells using the chimeric Gal4: PPARα system. Willson et al. (2000) has characterized the

TABLE 1
PPARα piperidine agonist structures and binding constants
Values are presented as mean ± S.E.M.

Compound	Structure	K_i		
		HsPPARα	HsPPARβ	HsPPARγ
		μM		
CP-865520		0.0740 ± 0.129	>10	>10
CP-775146		0.0245 ± 0.003[a]	>10	>10
CP-868388		0.0108 ± 0.002[b]	>10	3.47 ± 1.174

[a] $P < 0.05$ versus CP-865520 using the Student's t test.
[b] $P < 0.05$ versus CP-775146 using the Student's t test.

first generation fibrates (clofibrate, fenofibrate, and bezafibrate) in similar cell-based assays and found them to be weak agonists against all PPAR subtypes (EC_{50} values 20–500 μM) and with relatively low selectivity ratios (≤10–20-fold). As shown in Fig. 2A, the piperidine compounds showed robust, 60- to 70-fold induction of reporter gene activity and excellent potency with EC_{50} values of 219, 57, and 18 nM for '520, '146, and '388, respectively. Both '146 and '388 demonstrated statistically significantly improvements to potency versus '520 (Table 2) and is consistent with the rank ordering of the compounds based upon in vitro binding data. The piperidine agonists also demonstrate clear superiority over fenofibrate with respect to efficacy and nearly 23- to 280-fold improvement in potency.

We also evaluated these compounds for the ability to activate human PPARβ and PPARγ using the chimeric receptor system. The piperidine agonists demonstrated no activation of PPARβ and only poor activation of human PPARγ. Fenofibrate displayed minimal activity against PPARγ with respect to the doses achievable in this assay. No appreciable activity was achieved until dosing to 10 μM, thus resulting in EC_{50} values in the micromolar range. (Fig. 2B and Table 2). Whereas the binding data had suggested no appreciable

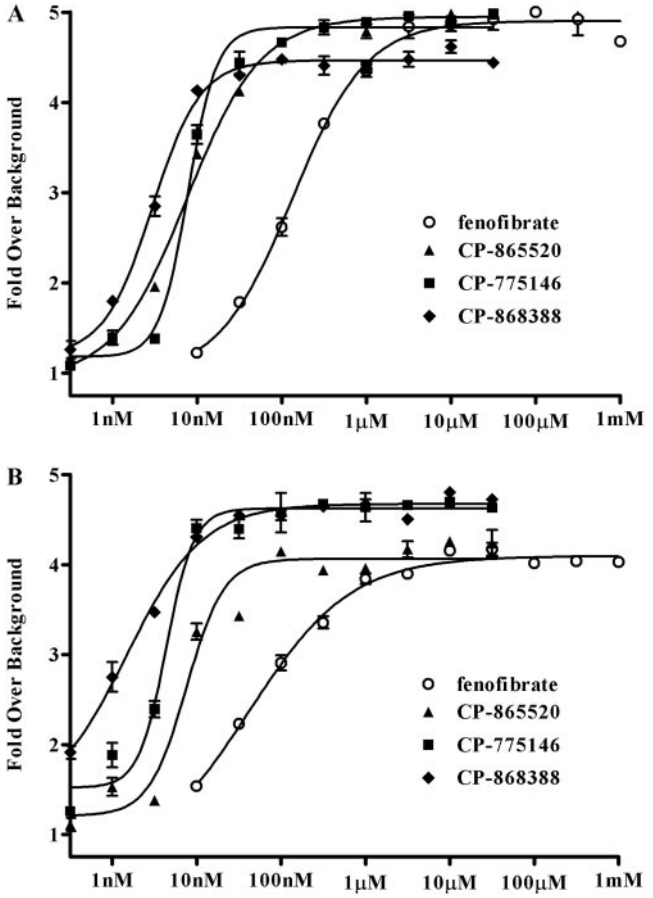


Fig. 1. Improved recruitment of cofactors by piperidine agonists. A, SRC-1 recruitment. Ligand-dependent recruitment of SRC-1 peptide was assessed in a fluorescence resonance energy transfer assay using GST:HsPPARα incubated in the presence of increasing concentrations of either fenofibrate or the piperidine agonists. The ratio of fluorescence signal obtained with bound versus unbound peptide was determined and plotted as -fold induction versus vehicle treatment. The piperidine agonists were significantly more potent than fenofibrate ($p < 0.00001$) in SRC-1 recruitment, whereas they did not show statistically significant differences in potency among themselves. B, PGC-1α recruitment. The study was performed as in A but used a PGC-1α peptide. The piperidine agonists were significantly more potent than fenofibrate ($p < 0.00001$) in PGC-1α recruitment, whereas they did not show statistically significant differences in potency among themselves.

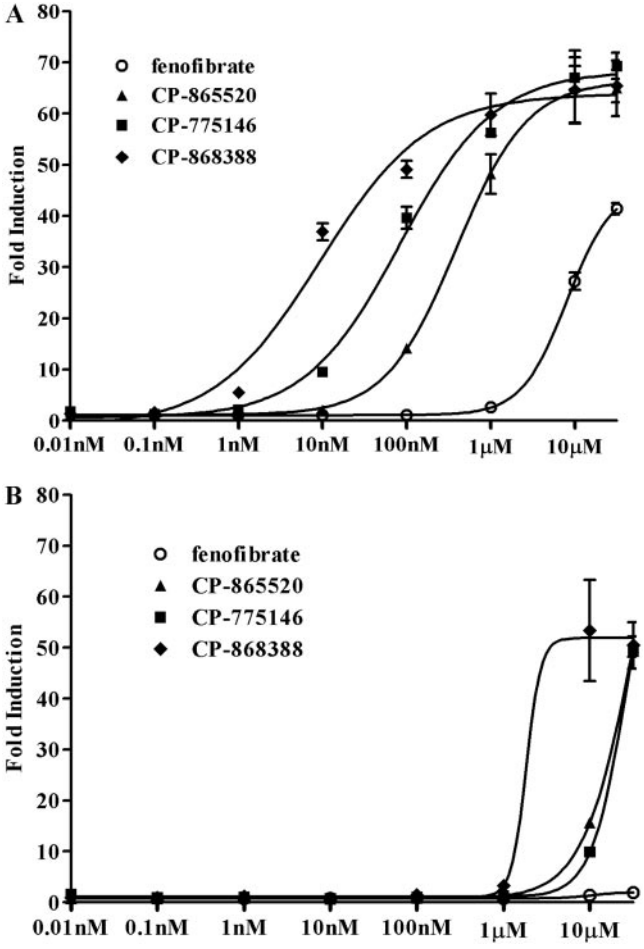


Fig. 2. Piperidine agonists demonstrate robust transcriptional activation of PPARα. A, potentiation of Hs:PPARα. HepG2 cells were transfected with a 5× Gal4 RE-luciferase reporter and a Gal4:HsPPARα expression vector and treated with increasing concentrations of either fenofibrate or the indicated piperidine agonist for 24 h. Luciferase activity was measured, corrected for transfection efficiency, and plotted as the -fold induction versus vehicle treatment. EC_{50} values and statistically significant differences are reported in Table 2. The piperidine agonists demonstrated statistically significant increase in potency versus fenofibrate ($P < 0.0004$). B, potentiation of Hs:PPARγ. The study was performed as in A but using a Gal4:HsPPARγ expression vector.

TABLE 2
Human and rodent PPAR transactivation (EC_{50}) values
Values are presented as mean ± S.E.M.

Receptor	CP-865520	CP-775146	CP-868388
		μM	
Hs:PPARα	0.219 ± 0.039	0.057 ± 0.039[a]	0.018 ± 0.039[b]
Rn:PPARα	0.573 ± 0.090	0.284 ± 0.101	0.030 ± 0.090[c]
Hs:PPARγ	7.550 ± 1.559	9.900 ± 1.559	1.425 ± 1.559[d]
Rn:PPARγ	>10	>10	9.330

[a] $P < 0.016$ versus CP-865520 for Hs:PPARα using ANOVA with a Bonferroni correction.
[b] $P < 0.005$ versus CP-865520 for Hs:PPARα using ANOVA with a Bonferroni correction.
[c] $P < 0.001$ versus CP-865520 Rn:PPARα using ANOVA with a Bonferroni correction.
[d] $P < 0.005$ versus CP-775146 for Hs:PPARγ using ANOVA with a Bonferroni correction.

PPARγ activity, in the cell-based assay '388 was found to have statistically significant improved potency toward PPARγ. The α/γ selectivity ratios generated with cell-based activity ranged from 34- to 173-fold. Thus, '520 and '146 function as selective PPARα agonists, whereas '388 functions as an α/γ dual agonist when dosed at greater than 1 μM in cell-based assays using human PPAR subtypes.

As a prerequisite to characterizing these compounds in vivo, we also assessed the selectivity of the piperidine agonists against the rodent PPARα and PPARγ receptors. The compounds demonstrated EC_{50} values for rat PPARα that were generally 2- to 4-fold lower than their human orthologs but with respectable potency ranging from 30- to 573-nM (Table 2). These compounds were also very poor activators of PPARγ as all EC_{50} values for rat PPARγ being greater than 10 μM. This was reflected with improved α/γ selectivity ratios of 17- to 310-fold. As such, all of the piperidine agonists function as PPARα-selective agonists in cell culture when tested in the context of the rodent receptors.

We next sought to further characterize the specificity of the piperidine agonists against other members of the human nuclear receptor superfamily. As shown in Fig. 3, the piperidine agonists failed to demonstrate transactivation potential against several representative members of the nuclear receptor family when dosed at 5 μM. In concordance with our earlier studies, these compounds are robust activators of human PPARα with approximately equal efficacy and demonstrate limited activity against PPARγ, and no activity against PPARβ. It is noteworthy that '388 demonstrated a statistically significant 5-fold increase of PPARγ transactivation potential versus '146 at the drug concentrations tested. These studies demonstrate the highly selective nature of the piperidine agonists for the PPAR subfamily.

Hypolipidemic Activity in Vivo. We next sought to characterize the ability of these compounds to modulate PPARα activity in vivo. A pharmacokinetic analysis of the piperidine compounds was performed to assess exposure with respect to the different chemical structures within the series. The orally administered piperidine agonists seemed to be well tolerated and demonstrated similar parameters for the highest drug concentration found in plasma, area under the curve, and protein binding (Table 3). Thus, the structural differences between the compounds did not appreciably alter bioavailability and allowed direct in vivo comparisons to be performed.

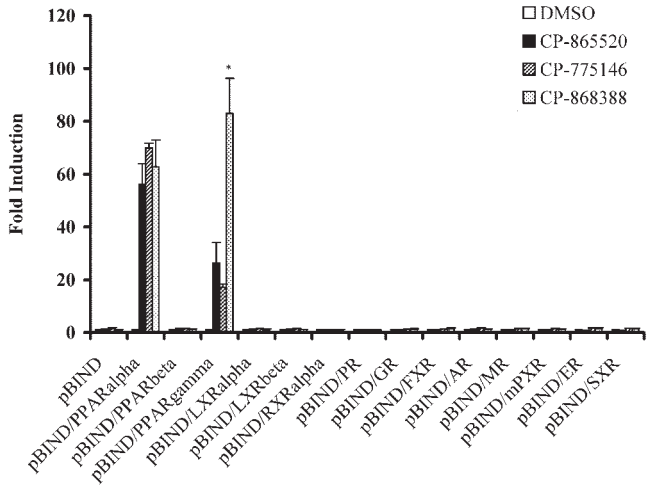


Fig. 3. The piperidine agonists induce selective activation of PPARα. HepG2 cells were transfected with the 5× Gal4 RE-luciferase reporter and the indicated Gal4 chimeras and treated with DMSO or the indicated piperidine agonists (5 μM) for 24 h. Luciferase activity was measured, corrected for transfection efficiency, and plotted as fold induction versus Gal4:PPARα treated with DMSO (normalized to 1). '388 was found to be significantly more efficacious than '146 (*$p < 0.016$).

Efficacy was next assessed in vivo by orally gavaging mice with either vehicle or piperidine agonist once daily for 2 days, followed by quantification of serum triglycerides 24 h after the final dose. This regimen provided the most reproducible triglyceride response during preliminary dose ranging and time course studies and thus was adopted for routine in vivo analysis (data not shown). As shown in Fig. 4, all three compounds demonstrated a robust and highly significant decrease in circulating plasma triglycerides. Triglyceride lowering was also dose-dependent with the greatest efficacy achieved at the 3.0 mg/kg dose, with triglyceride decreases of −80, −73, and −50% observed for '520, '146, and '388 (respectively). In contrast, fenofibrate dosed at 300 mg/kg for 21 days in mice demonstrated a moderate −28% decrease in serum triglycerides (Olivier et al., 1988), highlighting the improved hypolipidemic effect of the piperidine agonists. No statistically significant differences were found with respect to lipid lowering within the compound classes when queried at equivalent doses. These results demonstrate that the piperidine agonists have outstanding triglyceride lowering activity in vivo.

Piperidine Agonists Robustly Modulate Gene Expression in Wild-Type, but Not PPARα-Null Mice. Having observed potent triglyceride-lowering activity in vivo, we next sought to understand the gene expression networks that drive this activity. Wild-type or PPARα-null mice were treated for either 24 h (short term) or 5 days (long term) with the piperidine agonists to observe transcriptional responses of single and multiple dosing regimens in the murine liver. A 2.5-fold change and $P <= 0.05$ versus vehicle control was selected as the inclusion criteria and global changes to gene expression were visualized by hierarchical clustering shown in Fig. 5. Short-term treatment led to 202, 221, and 249 differentially expressed genes in the murine liver for '520, '146, and '388, respectively. Although '388 demonstrated statistically greater potency in cell-based assays and correlated with the greatest number of acutely modulated genes, this correlation disappears upon long-term dosing. Five-day dosing resulted in doubling of differentially expressed transcripts that is well balanced among the compounds (392, 392, 389 differentially expressed genes for '520, '146, and '388, respectively). It should be noted that the piperidine agonists demonstrate a profound ability to down-regulate approximately 50% of the differentially expressed genes in both the short- and long-term settings. There was a small subset of 30 genes within the 24-h cohort that were not differentially

TABLE 3
Oral pharmacokinetic parameters of PPARα piperidine agonists

Compound	C_{max}	AUC	F
	μg/ml	μg/h/ml	%
CP-865520	2.6	16	58
CP-775146	2.3	8	73
CP-868388	3.0	8	53

C_{max}, highest drug concentration found in plasma; AUC, area under the curve; F, bioavailability.

expressed at 5 days (Supplemental Table 1). The majority of these genes seem to be modulated indirectly, because only *ANGPTL4* has been shown to be a direct PPAR target gene (Ge et al., 2005). The transient modulation of these genes may reflect an early and indirect response to metabolic effects PPARα activation, such as induction of fatty acid oxidation. However, further studies will be required to verify these findings.

PPARα-null mice were also employed to evaluate the in vivo selectivity of the piperidine agonists. DeLuca et al. (2000) demonstrated that PPARβ and PPARγ ligands that lack PPARα activity in vitro can induce peroxisomal proliferation in PPARα-null mice, indicating that hepatic PPARβ and PPARγ activation is sufficient to induce PPARα target gene expression. Given the same inclusion criteria as for the wild-type mice, no differentially expressed genes were identified in response to piperidine agonist treatment. The threshold was then lowered to 1.05-fold change occurring in at least two of the three compound-treated groups. This strategy yielded a single gene (*ADRB2*) that was mildly induced at 24 h and 9 additional genes exhibiting variable modulation at 5 days (Supplemental Table 2). Previous studies using the PPARα agonist Wy14,643 in PPARα-null mice have identified Wy-14,643-dependent genes that are PPARα-independent. (Anderson et al., 2004a,b) Comparison of our PPARα-independent genes (Supplemental Table 2) with those of the previous studies demonstrated only one commonly modulated gene in the PPARα-null mice, *CYP2F1*. However, the directionality of this gene was different in that it was induced in our study and repressed in that by Anderson et al. (2004a). Overall, the failure of the piperidine agonists to induce know lipid catabolic genes, coupled with the absences of triglyceride lowering in the PPARα-null mice in response to treatment with the piperidine agonists (data not shown) demonstrated that these compounds exhibit excellent PPARα selective behavior in vivo.

The improved potency and selectivity of the piperidine agonists could provide additional pleiotropic effects that may prove to be unique to PPARα-selective series. For example, PPARα ligands have been associated with anti-inflammatory effects, glucose sensitization, and weight loss in preclinical models (Staels et al., 1998; Kockx et al., 1999; Fu et al., 2003; Ravnskjaer et al., 2005). Given that the liver is a primary site of action for PPARα and that it plays a central role in energy metabolism and physiology, we used Ingenuity Pathway Analysis to further scrutinize the gene expression networks induced by the piperidine agonists.

The canonical pathways exhibiting the most statistically significant changes in response to compound treatment are shown in Table 4. Fatty acid metabolism demonstrated the greatest number of modulated genes within a given pathway

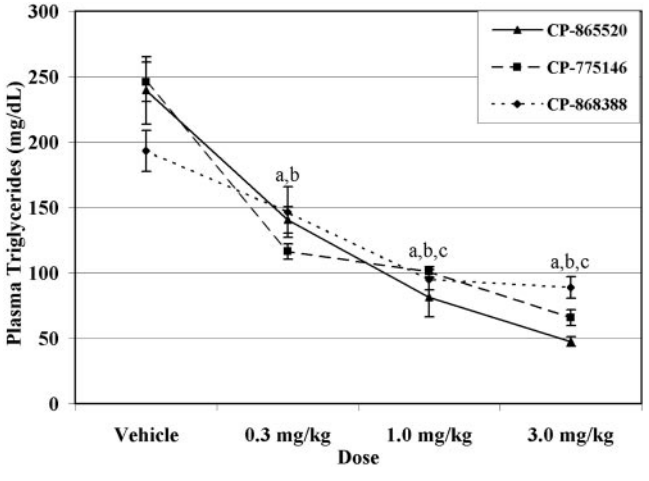


Fig. 4. The piperidine agonists demonstrate hypolipidemic activity in vivo. Male *B6/CBF1J* mice were orally gavaged with a single dose of vehicle or the indicated amount of the piperidine agonists. Mice were sacrificed 24 h later and plasma triglyceride levels were determined. Statistically significant lipid lowering versus vehicle were obtained for all compounds: ^{a} $p < 0.0004$ for '388, ^{b} $p < 0.0004$ for '520, ^{c} $p < .000000033$ for '146. No statistically significant differences were found between compounds when comparing lipid lowering at equivalent doses.

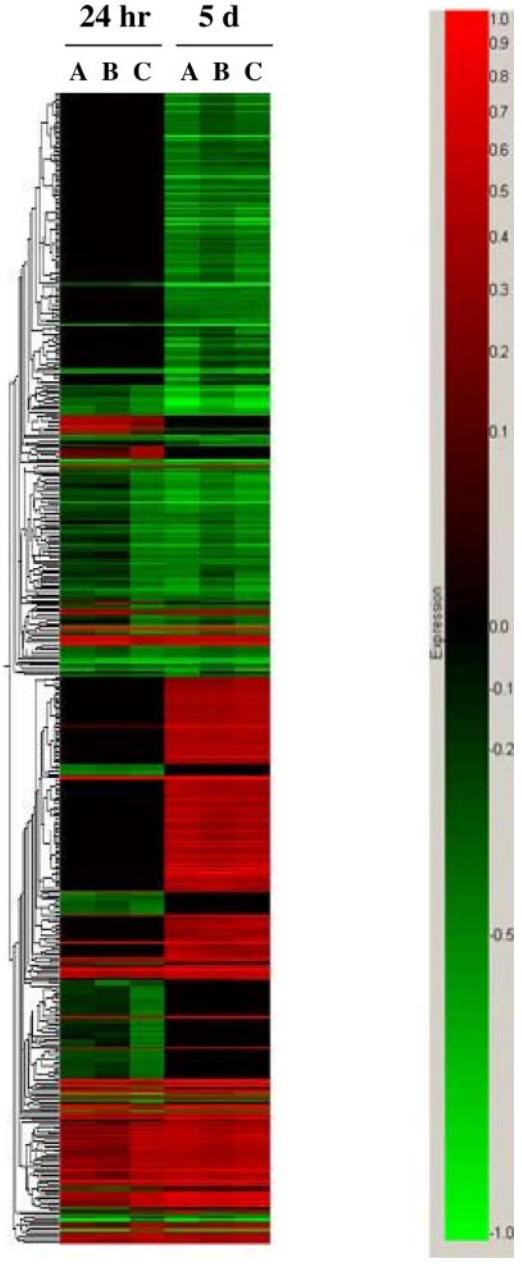


Fig. 5. The piperidine agonists induce robust gene expression changes in murine liver. Gene expression changes in murine liver were analyzed via hierarchical clustering and ordered as shown based upon the similarity of gene modulation. Treatments included '146 (A), '520 (B), or '388 (C) for either 24 h or 5 days. The expression ratio key (right) shows the magnitude of change for either induction (red) or repression (green).

at both the short- and long-term time points (ratio column, Table 4), as would be expected of a PPARα agonist. Additional pathways including tryptophan metabolism, valine-leucine-isoleucine degradation, LPS-IL1 mediated inhibition of RXR function, arachidonic acid metabolism, and fatty acid elongation in mitochondria could be considered subcategories of fatty acid metabolism based upon common genes involved in the metabolism of amino acids and lipid second messengers (Supplemental Table 3). Numerous genes previously identified as PPARα-responsive and involved in the metabolism of long-chain fatty acids (*ACAA1B*, *ACOT1*, *ACOT8, ADRP*, *EHHADH*, *ME1*, *SLC22A5*, *SLC25A20*) and peroxisomal proliferation (*ACOX1*, *PEX11A*) were found to be significantly induced on the array and are listed in Supplemental Table 4. The modulation of these direct target genes as well as other PPARα-responsive genes were verified using quantitative real-time PCR and in all cases were found to confirm the magnitude and directionality observed in the microarray study (Supplemental Table 4).

Pathways involved with nitrogen metabolism and protein ubiquitination were also differentially expressed (Supplemental Table 3). We observed down-regulation of nitrogen metabolism [carbonic anhydrases and carbamoyl-phosphate synthetase (*CPS1*), the rate-limiting enzyme of urea synthesis] genes with a concomitant induction of genes from the ubiquitination pathway (heat shock proteins, 20S and 26S proteosome). Modulation of these pathways is in agreement with the repression of urea cycle enzymes, including CPS1 (Kersten et al., 2001) and the induction of proteome maintenance genes by Wy-14,643 in mice (Anderson et al., 2004b).

PPARα seems to play a special anti-inflammatory role in the liver, where fibrates have been shown to inhibit the acute phase response (APR) in rodent liver as well as in man (Staels et al., 1998; Kockx et al., 1999). An expected finding was the significant impact that the PPARα-selective piperidine agonists had upon the APR and the coagulation and complement cascades (Supplemental Table 3). Most of these genes were modestly down-regulated 2- to 3-fold and would be predicted to be anti-inflammatory and antithrombotic in nature. Down-regulation of additional genes mapping to cellular functions of immune response, cell-to-cell signaling, and interaction were also observed (Supplemental Table 5). Suppressed genes included extracellular matrix proteins or adhesion molecules such as *CDH2*, *CEACAM1*, *SDC2*, *SDC4*, and *VTN* or transmembrane signaling molecules such as CXCL12, and MST1.

We also examined genes involved in carbohydrate metabolism and found significant alterations in several key genes, including *PDK4*, *SLC2A2*, and *SLC37A1* (Supplemental Table 6). With the exception of PDK, which was induced 6- to 26-fold, many of the glucose metabolism genes were also down-regulated. PDK4 induction mediated by PPARα agonists has been previously identified (Sugden et al., 2001; Yu et al., 2001) and would be predicted to inhibit the decarboxylation of oxaloacetate catalyzed by the pyruvate dehydrogenase complex and therefore block the oxidation of glucose. In addition, the down-regulation of SLC37A1 (the glucose 6-phosphate transporter) and SLC2A2 (the bidirectional glucose transporter) would be postulated to diminish the net glucose efflux from the liver. These trends are consistent with the potential of PPARα activation to promote metabolic fuel source exchange between glucose and fatty acids.

Discussion

The present study reports the characterization of a novel class of PPARα agonists that present potent and selective behavior in vitro and robust lipid-lowering activity and gene modulation potential in vivo. It is noteworthy that improvements in potency and selectivity could enhance the anti-inflammatory potential of this mechanism or lead to the discovery of unappreciated and therapeutically beneficial pathways in addition to their recognized lipid lowering activity in vivo.

To test this idea directly, we used pathway mining of the transcriptional networks induced by the piperidine agonists in the murine liver after short- and long-term exposure. As was anticipated, lipid metabolism was the most significantly induced function at both the short- and long-term time points. Direct and indirect target genes modulated by compound treatment are shown in Fig. 6 and in Supplemental Table 7. Robust induction of genes involved in lipid trafficking (*ABCD2*, *ABCG2*, *CD36*, *CRAT*, *FABP2*, *FABP3*, *FABP4*, *MTTP*, *PCTP*, *SLC27A1*), lipid mobilization (*ADFP*, *CIDEA*, *MGLL*) and mitochondrial and peroxisomal β-oxidation

TABLE 4

Top canonical pathways expressed in response to piperidine agonists in wild-type mice

Ratio represents the number of differentially expressed genes (>1.5-fold) relative to the number of genes comprising a given canonical pathway. *P* values for the ratios obtained with each compound treatment using the Fisher's exact test.

Common Canonical Pathway	24 h				5 days			
	Ratio	CP-865520	CP-775146	CP-868388	Ratio	CP-865520	CP-775146	CP-868388
Fatty acid metabolism	21/187	2.29×10^{-15}	1.25×10^{-14}	2.43×10^{-13}	28/187	3.27×10^{-16}	3.52×10^{-16}	2.61×10^{-16}
Tryptophan metabolism	14/237	9.24×10^{-8}	3.21×10^{-8}	2.05×10^{-7}	19/237	6.03×10^{-9}	6.32×10^{-9}	5.23×10^{-9}
Nitrogen metabolism	8/133	5.00×10^{-8}	9.55×10^{-8}	2.99×10^{-7}	10/133	4.23×10^{-8}	4.35×10^{-8}	3.90×10^{-8}
Coagulation system	8/35	1.07×10^{-7}	2.04×10^{-7}	6.33×10^{-7}	10/35	1.10×10^{-7}	1.13×10^{-7}	1.02×10^{-7}
Valine, leucine and isoleucine degradation	11/107	4.95×10^{-9}	1.18×10^{-8}	5.50×10^{-8}	11/107	3.22×10^{-6}	3.31×10^{-6}	2.97×10^{-6}
Acute phase response	14/172	4.07×10^{-6}	1.91×10^{-6}	1.07×10^{-5}	23/172	2.37×10^{-9}	2.50×10^{-9}	2.00×10^{-9}
LPS/IL-1 mediated inhibition of RXR function	16/195	4.19×10^{-6}	4.29×10^{-7}	3.08×10^{-6}	21/195	7.89×10^{-7}	8.27×10^{-7}	6.85×10^{-7}
Arachidonic acid metabolism	10/211	2.10×10^{-5}	4.30×10^{-5}	1.48×10^{-4}	16/211	5.96×10^{-7}	6.20×10^{-7}	5.31×10^{-7}
Fatty acid elongation in mitochondria	6/45	1.69×10^{-7}	2.77×10^{-7}	6.65×10^{-7}	6/45	7.16×10^{-6}	7.28×10^{-6}	6.81×10^{-6}
Protein ubiquitination pathway	7/203	2.85×10^{-2}	1.31×10^{-2}	8.90×10^{-3}	18/203	3.48×10^{-7}	3.63×10^{-7}	3.06×10^{-7}
Complement system	5/36	2.00×10^{-4}	2.95×10^{-4}	5.83×10^{-4}	8/36	6.45×10^{-6}	6.58×10^{-6}	6.05×10^{-6}

LPS/IL-1, lipopolysaccharide/interleukin-1; RXR, retinoid X receptor.

(*ACAA1*, *ACADL*, *ACADM*, *ACOT1*, *ACOT8*, *ACOX1*, *DCI*, *ECH1*, *EHHADH*, *HADHB*, *PECI*) were observed. Clearly, the piperidine agonists are inducing fatty acid catabolic pathways that lead to statistically significant reduction in serum triglycerides in vivo. The improved potency of these compounds is also reflected in that triglyceride lowering was observed at the lowest dose (0.3 mg/kg) tested.

Studies using PPARα-null mice and potent inflammatory mediators such as LTB4 and LPS demonstrate that this nuclear receptor functions as a general modulator of the inflammatory response (Devchand et al., 1996; Delerive et al., 1999). To our knowledge, this is one of the first studies using a selective PPARα agonist to demonstrate anti-inflammatory potential in the liver. Treatment with the piperidine agonists markedly down-regulated several the so-called "positive" genes of the APR including *C2*, *C5*, *C9*, *C4A*, *F2*, *FGB*, *ITIH4*, *KLKB1*, *MBL2*, *PLG*, *SERPINA3*, *SERPINF2*, and *SERPING1*. Additional APR products *SAA1*, *SAA2*, and *AGT* are also repressed. Induction of the positive APR products occurs through activation of the NFκB, CEBPβ, and STAT3

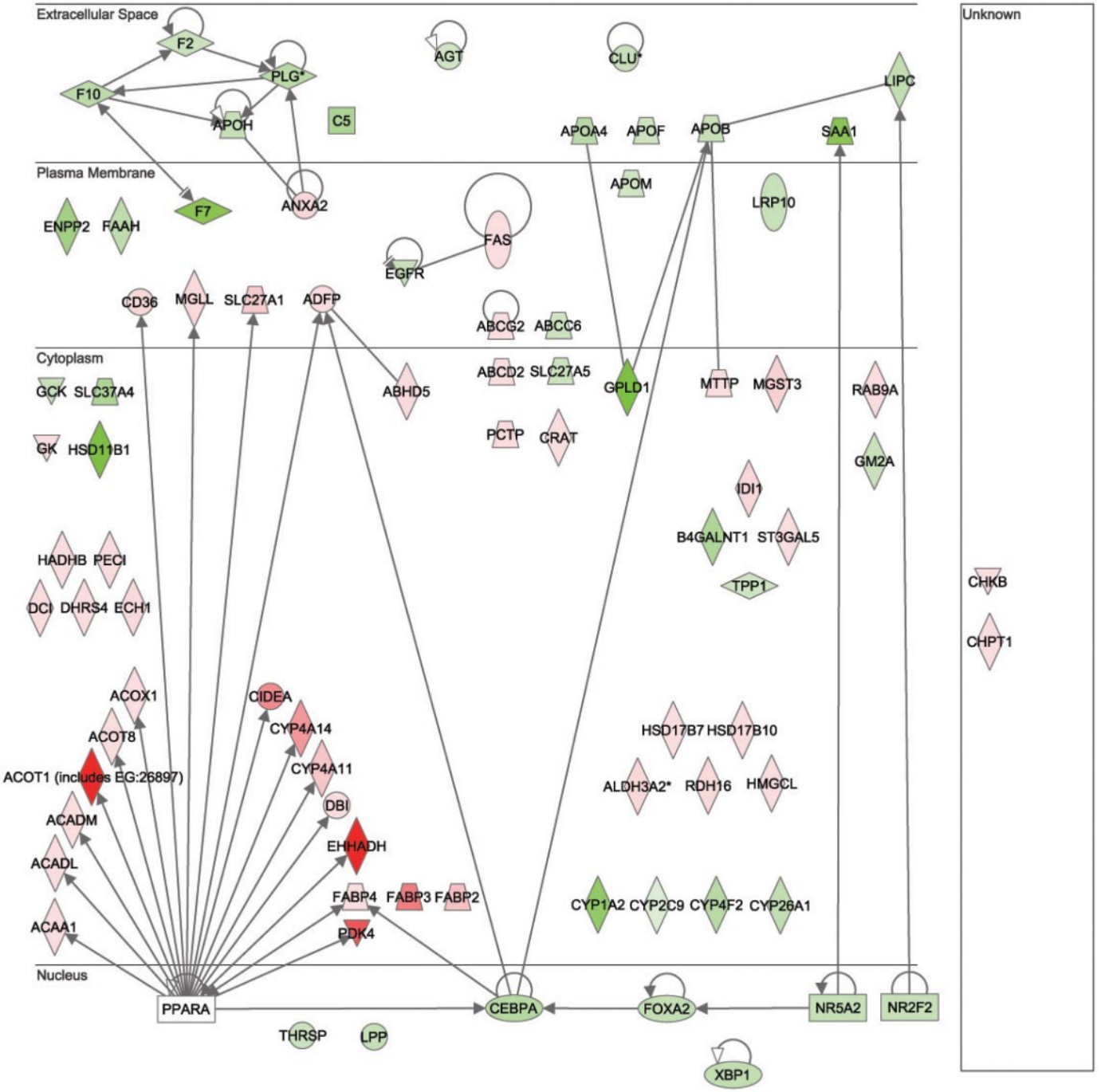


Fig. 6. Lipid metabolism genes modulated by the piperidine agonists. Direct and indirect PPARα target genes involved in lipid metabolism are depicted in their subcellular compartments and shaded with respect to expression changes in response to compound treatment. Induced genes are depicted in red, and down-regulated genes are in green with shading intensity proportional to the magnitude of change. Lines connecting nodes represent a direct interaction corresponding to either an effect on expression or protein-protein interactions. Supplemental Table 7 contains additional annotation for the genes found in this figure.

pathways in response to inflammatory cytokines. It is noteworthy that the fibrates have been shown to blockade inflammatory cytokine signaling mediated by interleukin-1, interleukin-6, and tumor necrosis factor α via transrepression of nuclear factor-κB, C/EBP, activator protein-1, and signal transducer and activator of transcription (Staels et al., 1998; Marx et al., 1999; Devchand et al., 2004; Zambon et al., 2006). Down-regulation of APR genes in our studies suggests that the piperidine agonists can also transrepress the action of nuclear factor-κB, C/EBP β, and STAT3 in vivo.

In addition, multiple genes involved in the immune response (*CXCL12*, *LIFR*, and *MST1*) and cell binding (*CDH2*, *CEACAM1*, *SDC2*, *SDC4*, *VTN*) were also repressed in response to these agonists (Supplemental Table 3). Electronic Northern analysis suggests that these gene products are generally restricted to the liver. However, this method does not have sufficient resolution to determine whether these transcripts are derived from hepatocytes or Kupffer cells. Under normal metabolic conditions, PPARα is either not expressed in the murine Kupffer cell or is expressed at levels beneath detection (Gonzalez, 2002). This would suggest that the immune-related transcripts are originating in the hepatocyte. Suppression of hepatic adhesion molecule expression may represent an unappreciated and unique anti-inflammatory mechanism associated with PPARα activation in the liver. There is precedence for this type of anti-inflammatory mechanism in endothelial cells. PPARα activation has been shown to decrease cytokine-mediated expression of the adhesion molecule VCAM-1 in endothelial cells and thus reduces the adhesion of monocytes in the vasculature (Marx et al., 1999).

PPARα is also implicated in hepatic glucose homeostasis and insulin sensitivity and secretion, in part by decreasing the expression of gluconeogenic enzymes (Chou et al., 2002), altering substrate utilization for hepatic glucose production (Xu et al., 2002), and the alleviation of hepatic lipotoxicity (Lalloyer et al., 2006). Our studies suggest that the piperidine agonists can suppress hepatic glucose production by these and other mechanisms. Down-regulation of SLC37A4 would prevent the transport of glucose 6-phosphate (g-6-P) to the lumen of the endoplasmic reticulum, where it is dephosphorylated to allow glucose secretion. Sequestration of g-6-P in the cytoplasm will promote glycogen synthesis and storage, as is demonstrated by human genetic errors targeting *SLC37A4*. Repression of the facultative glucose transporter SLC2A2 would also hamper the transport of free glucose (Guillam et al., 1997). The robust induction of PDK4, a key negative mediator of glucose oxidation, is an additional mechanism to preserve hepatic glucose. PDK4 inactivation of the pyruvate dehydrogenase complex inhibits glucose oxidation and promotes fatty acid oxidation. PDK4 has previously been shown to be induced by Wy14,643 in both muscle and liver (Motojima and Seto, 2003) but PDK4 does not seem to be a direct PPARα target gene (Degenhardt et al., 2007). Nevertheless, our studies demonstrate a major induction of PDK4, implying an overall propensity to conserve hepatic glucose during PPARα activation.

Although the therapeutic potential of PPAR modulators has appeared promising during preclinical development, the field has been unable to reduce this to practice in humans. For example, Terra et al. (2008) evaluated a selective PPARα agonist (CP-7788752; Hamanaka and Kehrli, 2005) with biochemical properties that are very similar to the piperidine series. CP-778875 is a high-affinity PPARα ligand (IC_{50} = 7 nM) that is >100-fold selective for PPARβ and >1000-fold selective with PPARγ. It has recently been assessed in a 6-week phase II trial in subjects with mixed dyslipidemia and type 2 diabetes. Although this compound significantly reduced fasting triglycerides and elevated HDL, there were no significant changes in either fasting glucose or hemoglobin A_{1c}. In addition, anticipated anti-inflammatory effects were not observed. The failure to affect all endpoints (lipid, glucose, and anti-inflammatory) in the diabetic cohort may suggest that certain patient populations, such as those featuring the metabolic syndrome, may gain greater benefit from a selective PPARα mechanism. Post hoc analysis of several fibrate studies in populations of overweight patients with high plasma triglycerides and low levels of HDL derive a disproportionately large reduction in cardiovascular events when treated with these agents (Barter and Rye, 2008). Nevertheless, we must question why only a subset of the pharmacological endpoints expected from preclinical evaluation was actually observed in the patient population studied?

Species-dependent differences in direct PPARα target genes could be a fundamental reason for differences in physiology between preclinical in vivo models such as rodents and primates. ACOX1, the rate limiting step of peroxisomal β-oxidation, is a key example of a rodent-specific (mice and rats) PPARα target gene that is not directly modulated in primates because of differences in the peroxisome proliferator response element between species (Kane et al., 2006). Rodents treated with PPARα agonists demonstrate the induction of ACOX1 and subsequent peroxisomal proliferation and oxidative damage leading to pathologies in the liver, skeletal muscle, and heart (Klaunig et al., 2003; Pruimboom-Brees et al., 2006). However, this is not recapitulated in the primate, and the capacity for induction of *ACOX1* and other genes involved in peroxisomal β-oxidation seem to be an order of magnitude lower in primates than in rodents (Cariello et al., 2005). This is corroborated by lack of cardiac necrosis as assessed by cardiac troponin I measurements in the CP-778875 clinical trial (Terra et al., 2008).

PPARα-dependent repression of immune and carbohydrate pathways seems to be mediated by a transrepression mechanism and could also differ in a species- or disease-dependent manner. Sequestration of coactivators, including SRC-1 and SRC-3/pCAF, has been shown to dramatically impair the transcriptional activity of NFκB (Sheppard et al., 1999), whereas titration of SRC-2 by activated PPARα will negatively interfere with CEBPβ (Gervois et al., 2001). The piperidine agonists are shown to be strong recruiters of SRC-1 and PGC-1α, and additional coactivators, including SRC-2, SRC-3, and PGC-1β (Fig. 2, data not shown). Therefore, the piperidine agonists are likely to transrepress NFκB, CEPBβ, and potentially other inflammatory mediators in part by coactivator competition. Coactivator levels will fluctuate in response to environmental factors such as temperature and nutritional status (Puigserver et al., 1998; Yoon et al., 2001). As such, the clinical translation of a "squelching" transrepression mechanism may be highly variable and difficult to recapitulate if it is dependent on such factors as the model organism (rodent, primate, human), underlying pathology, diet, and duration of treatment.

In conclusion, the piperidine series represents a novel class

of PPARα agonists that present potent and selective behavior in vitro and robust lipid-lowering activity and gene modulation potential in vivo. The transcriptional networks activated by these selective agents are consistent with PPARα-mediated induction of fatty acid oxidation and are suggestive of anti-inflammatory action correlating with suppression of the acute phase response. The anti-inflammatory response is especially intriguing, given its emerging role in the etiology of metabolic disease. However, it should be noted that no novel PPARα selective agents have successfully completed advanced clinical trials and that most trials have terminated because of safety concerns. Therefore, it will be of interest to perform additional studies with long-term dosing of piperidine agonists in preclinical models of atherosclerosis and metabolic syndrome to directly assess their therapeutic potential and translation in these disease states as well as thoroughly assess their safety profile using multiple in vitro and in vivo models.

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