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Comparative Evaluation of Gemcabene and PPAR Ligands in Transcriptional Assays of Peroxisome Proliferator-Activated Receptors: Implication for the Treatment of Hyperlipidemia and Cardiovascular Disease

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Abstract

Gemcabene, a late-stage clinical candidate, has shown efficacy for LDL-C, non-HDL cholesterol, apoB, triglycerides and hsCRP reduction, all risk factors for cardiovascular disease (CVD). In rodents, gemcabene showed changes in targets, including apoC-III, apoA-I, peroxisomal enzymes, considered regulated via PPAR gene activation, suggesting a PPAR-mediated mechanism of action for the observed hypolipidemic effects observed in rodents and humans. In the current study, the gemcabene agonist activity against PPAR subtypes of human, rat and mouse were compared to known lipid lowering PPAR activators. Surprisingly, gemcabene showed no or little PPAR-α transactivation compared with reference agonists, which showed concentration-dependent transactivation against human PPAR-α of 2.4 to 30-fold (fenofibric acid), 17-fold (GW590735), and 2.3 to 25-fold (WY14643). These agents also showed robust transactivation of mouse and rat PPAR-α in a concentration-dependent manner. The known PPAR-δ agonists, GW1516, L165041 and GW0742, showed potent agonist activity against human, mouse and rat receptors (ranging from 165- to 396-fold). In contrast, gemcabene at the highest concentration tested (300 µM) showed no response in mouse and rat and a marginal response against human PPAR-δ receptors (3.2-fold). For PPAR-γ, gemcabene showed no agonist activity against all 3 species at 100 µM and marginal activity (3.6-5 fold) at 300 µM. In contrast, the known agonists, rosiglitazone, indomethacin and muraglitazar showed strong activation against the mouse, rat and human PPAR-γ receptors. No clear antagonist activity was observed with gemcabene against any PPAR-subtypes for all 3 species over a wide range of

concentrations. In summary, the transactivation studies rule out gemcabene as a direct agonist or antagonist of PPAR-α, PPAR-γ, and PPAR-δ receptors of these three species. These data suggest that the peroxisomal effects observed in rodents and the lipid regulating effects observed in rodents and humans are not related to a direct activation of PPAR receptors by gemcabene.

Suggested key words: gemcabene, PPAR-α, PPAR-δ, PPAR-γ, transactivation, nuclear hormone receptors.

INTRODUCTION

Cardiovascular diseases (CVD) constitute a broad number of conditions including heart and vascular disease, atherosclerosis, stroke and hypertension, and are the leading cause of global morbidity and mortality (1,2). Both genetic and environmental factors contribute to dyslipidemia and type 2 diabetes and can increase the risk for CVD (1,3-5). The discovery that Peroxisome Proliferator Activated Receptors (PPARs) are key regulators of metabolic pathways has led to significant research, drug discovery and understanding of the mechanisms of action of PPAR receptors and their implications for the prevention and treatment of metabolic disorders and CVD (6-9). Ligand-activated nuclear receptors have been exploited as treatments for the regulation of lipid and glucose metabolism to treat and reduce the risk of diabetes and CVD (4,8-10).

PPARs are ligand-activated transcription factors belonging to the nuclear receptor super family (11). PPARs are type II nuclear receptors containing a cysteine-rich Zn finger–motif DNA binding domain (12,13). The subtypes PPARα, PPARγ, and PPARδ (also known as PPARβ) (14) differ with respect to their tissue distribution and distinct roles in glucose and lipid

homeostasis (15-19) and energy homeostasis (19-22), as well as in other cellular functions (23-30). PPAR-α receptors are involved in ensuring energy availability during fasting with a key role in starvation (31). Their state of activation in the liver is regulated by dietary fatty acids and during fasting by fatty acids generated *via de novo* lipogenesis (32-34). In humans, these receptors play a broader role in lipid metabolism (32,35), regulating apolipoprotein (apo) genes such as apoA-I, apoA-II, apoA-V and apoC-III, fatty acid ß-oxidation genes like acyl-CoA oxidase, CPT-I and CPT-II, and fatty acyl CoA desaturase genes (*e.g.*, delta-6-desaturase) involved in fatty acid interconversion (36,37), phospholipid transfer protein (PLTP) involved in HDL metabolism and HMGCoA synthase 2 (HMGCS2) involved in ketone body synthesis (36,37). Many physiological effects of PPAR-δ activators overlap with those of PPAR-α activators.

Once the PPAR nuclear receptors were molecularly characterized and it was realized that fibrates and thiazolidinediones are PPAR ligands, an enormous effort was made within the pharmaceutical industry to create a large number of synthetic, specific and potent direct PPAR activating molecules. However, there are also natural physiological ligands that can trigger PPAR activation. For instance, there is a natural importance for providing fatty acid ligands to activate PPAR-α (31,32,34), thereby converting energy balance from a lipogenic to a ketogenic state in extended fasting periods.

Gemcabene lipid regulating activities were discovered following screening of small molecules with similar chemical functionalities for triglyceride lowering and other lipid regulating activities (i.e., HDL-C elevation) in chow-fed Sprague-Dawley rats (38). Structurally, gemcabene is a fraudulent fatty acid with two terminal gem-dimethyl carboxylate moieties (Figure 1). Although gemcabene dose-dependently increases liver weight and expression of hepatic peroxisomal enzymes in rats (38), there is insufficient data to conclude direct activation of the PPAR-α receptor by gemcabene. In chow-fed Sprague-Dawley rats, gemcabene dose-dependently reduced hepatic apoC-III mRNA levels, plasma triglycerides, LDL-C, VLDL-C, apoC-II, apoC-III, and apoB and elevated HDL-C, apoA-I and apoE (38).

In hypertriglyceridemic patients (TG≥200 mg/dL) with low HDL-C (HDL-C<35mg/dL) and normal LDL-C levels, gemcabene lowered plasma apoC-III, triglycerides, LDL-C, non-HDL-C, apoB and apoE, and elevated apoA-I, apoA-II and HDL-C (39). In a placebo-controlled double-blind clinical study in hypercholesterolemic patients on background statin therapy, gemcabene further reduced LDL-C, apoB, non-HDL-C and C-reactive protein (40). Since gemcabene showed increased peroxisomal activity in rats and modulated some of the known hepatic PPAR-α responsive genes (38), we evaluated the potential involvement of PPAR subtype activities by transactivation assays in PPAR subtype constructs. We present below our data demonstrating that gemcabene lacks significant activities against any of the PPAR subtypes for either mouse, rat or human receptors.

METHODS

Plasmid and Transactivation Assay

This study was commissioned to and conducted by Indigo Biosciences, State College, PA, 16801, USA. The Nuclear Receptor Reporter Cells utilized were available from Indigo Biosciences, a proprietary cell line expressing a hybrid receptor comprising the N-terminal Gal4 DNA-binding domain fused to the ligand-binding domain (LBD) of the specific nuclear receptor. The reporter gene, firefly luciferase, is functionally linked to the Gal4 upstream activation sequence (UAS). Descriptive information on reference compounds (known drugs and clinical candidates) used for assay validation or comparative purposes are displayed in Figure 1 and Table 1. The Nuclear Receptor assays were performed in three steps as described below.

In step 1, as shown in Figure 2, a suspension of Reporter Cells was prepared in Cell Recovery Medium (CRM) containing 10% charcoal-stripped fetal bovine serum (FBS). For antagonist assays, reporter cells were first supplemented with 2x-EC_{80} concentration of the appropriate reference agonist (see Table 1). Then, 100 µl of the Reporter Cell suspension treated with or without 2x-EC_{80} was dispensed into wells in a white 96-well assay plate. In step 2, prior to assay setup, test compounds were diluted using compound screening medium (CSM) containing 10% charcoal-stripped FBS to generate "2x-concentration" treatment media. Treatment medium (100 µl of each) was dispensed into wells pre-dispensed with the Reporter Cells. The assay was conducted in triplicate. Assay plates were incubated at 37 ^{o}C for 24 h. Finally, in Step 3, following the 24 h incubation period, treatment media were discarded and the

wells were rinsed once with Live Cell Multiplex Assay (LCMA) Buffer and subsequently treated with LCMA substrate. Following incubation at 37 ^{o}C for 45 min, fluorescence was measured to determine the relative number of live cells expressed as relative fluorescence unit (RFU) per assay well. The percent live cells were taken into consideration in the calculation of EC_{50}. Some of the reference agents were toxic to the cells and they have been mentioned in the comments section of Table-2. LCMA substrate was then discarded and 100 µl/well of Luciferase Detection Reagent was added. RLUs were quantified from each assay well to determine PPAR agonist and antagonist activities.

Assay Validation

Reference compounds (Table 1 and Figure 1) were utilized to confirm the performance of the specific lot of PPAR Reporter Cells. Reference compounds and gemcabene were assayed simultaneously to insure comparability (see individual data sets for identity and treatment concentration ranges of reference compounds). A vehicle control allowed determination of background activity in the assay and to calculate values of fold-activation and percent-inhibition of receptor activity.

Graphical Data Methods

Dose-response curve (DRC) analyses of the tested agents were performed *via* non-linear curve-fitting of fold-activation *vs.* log[Compound concentration] for PPAR *agonist* assays. Percent inhibition *vs.* log[Gemcabene concentration] for PPAR antagonist assays were calculated using GraphPad Prism software.

RESULTS

To determine if gemcabene is a direct activator of PPAR subtypes, transactivation assays were performed in Chinese Hamster Ovary (CHO) cells in both agonist and antagonist mode using mouse, rat and human PPAR subtype constructs. In this assay, activation of Gal4 expression induces luciferase activity, a measure of extent of PPAR gene activation. PPAR transcriptional activation values are presented in Table 2.

The results of PPAR-α activation in human, rat, and mouse are shown in Figure 3. Two lots of a potent PPAR-α agonist, GW590735, were tested for their PPAR-α agonist activities and, as expected, both showed potent agonist activity with an EC_{50} of 2.3 nM against human, 98 nM against mouse, and 2.2 µM against rat PPAR-α. WY-14643, a widely used PPAR-α activator reference agent (41,42), showed PPAR-α activation (EC_{50}) against human (32.6 µM), mouse (18.6 µM), and rat (10.4 µM) PPAR-α. The PPAR-α agonist GW590735 (43) showed robust maximal activation of 17-, 89-, and 83-fold against human, mouse, and rat PPAR-α, respectively (Figure 3), while gemfibrozil showed similar values to the originally reported EC_{50} of 193 µM (44). Muraglitazar, with dual PPAR-α/PPAR-γ agonist activity (45), was used to evaluate the robustness of this assay and to ensure that the assay can detect simultaneous activation of multiple PPAR subtypes for dual or pan agonists. In this assay, the dual PPAR-α/-γ agonist muraglitazar showed agonist activity against human PPAR-α and PPAR-γ, but not against mouse and rat PPAR-α, consistent with the literature (45).

Under the assay conditions and construct used, gemcabene showed essentially little or no PPAR-α activity against rat and mouse, but low marginal PPAR-α activity against human at the highest concentration (300 µM) tested (Figure 3).

Further, two lots of the potent PPAR-δ agonist GW0742 (21,46) were tested, and both gave identical activation values (Figure 3) with an EC_{50} of 0.0019 µM against human, and 0.036 µM and 0.059 µM against mouse and rat PPAR-δ, respectively (Table 2). PPAR-δ agonist L165041 (44,47,48) showed potent PPAR-δ agonist activity against human PPAR-δ, with an EC_{50} of 0.0170 µM, but less activation against the mouse PPAR-δ (EC_{50} of 0.630 µM). Similarly, the reference compound GW501516, widely studied for its hypolipidemic activities (49,50), showed potent PPAR-δ agonist activity with an EC_{50} of 0.0016 µM against human and EC_{50} of 0.044 µM and 0.063 µM against mouse and rat PPAR-δ, respectively (Figure 3 and Table 2). In conclusion, PPAR-δ reference compounds GW1516, L165041 and GW0742 confirm their robust agonist activity against human, mouse and rat PPAR-δ with maximal activation in the range of 165- to 396-fold. In contrast, gemcabene showed no activation up to 300 µM concentration against rat or mouse PPAR-δ, and marginal activation against human PPAR-δ only at the highest concentration tested. This clearly shows that gemcabene does not possess any direct PPAR-δ agonist activity under the assay conditions, while known PPAR-δ agonists showed expected level of agonist activities in a concentration-dependent manner.

As displayed in Table 2 and Figure 3, known PPAR-γ activators showed expected activity in this assay. Gemcabene did not show concentration-dependent activity up to 100 µM concentration, in the 3 species tested. However, rodent and human PPAR-γ receptors showed relatively marginal (3.6-5 fold) activation at (300 µM).

Since PPARs may influence lipid and carbohydrate metabolism through antagonist activity (51), we tested if gemcabene has antagonist activity against PPAR subtypes in human, rat, and mouse. As shown in Figure 4, gemcabene did not display antagonist activity against any PPAR subtypes, whereas known PPAR subtype antagonists (51,52) showed expected activities.

DISCUSSION

The present study evaluated gemcabene as a direct agonist/antagonist for the PPAR receptor subtypes in three species: rat, mouse, and human, in order to corroborate the earlier physiological findings of gemcabene-induced modulation of a subset of PPAR-α responsive genes in rodents (38). Specifically, we sought to determine whether the observed biological effects were a direct or indirect result of gemcabene activation of PPAR-α-related genes.

In the current study, while the reference compounds showed significant agonist activity in human, mouse and rat towards PPAR subtypes in transactivation assays, gemcabene essentially lacked agonist activity against rat and mouse PPAR-α up to the highest concentration tested (300µM). Unlike gemcabene, the reference PPAR-α activators showed robust concentration-

196 dependent transactivation against human PPAR-α ranging from 2.4-fold to 30-fold for fenofibric
197 acid (20) and 2.3 to 25-fold for WY14643 (29) (Table 2). These reference agents also showed
198 marked concentration-dependent transactivation for mouse and rat PPAR-α. Muraglitazar, a dual
199 PPAR-α and -γ agonist (45), showed 19- and 17-fold activation of human PPAR-α and PPAR-γ,
200 respectively (Table 2). The PPAR-α agonist GW590735 (43) also showed robust maximal
201 activation of 17-, 89-, and 83-fold against human, mouse and rat PPAR-α, respectively (Table 2).
202 These findings suggest that gemcabene lacks direct rat and mouse PPAR-α activation and is an
203 extremely weak direct agonist for the human PPAR-α receptor. Given the central role of PPAR-α
204 in lipid metabolism (7,11,13), it is possible and likely that gemcabene treatment in rodents causes
205 an indirect PPAR-α activation by endogenous cellular metabolites (27,33), as observed during
206 fasting and starvation (31).

207 Recognized PPAR-δ agonists L165041 (44,47,48), GW0742 (21,46) and GW1516 (49,50)
208 showed potent agonist activity against human, mouse and rat PPARs with maximal activation in
209 the range of 165- to 396-fold (Table 2). In this experiment, gemcabene showed little or no
210 activity and lacked a dose-response for PPAR-δ agonist activity against all species tested.

211 Finally, we investigated PPAR-γ agonist activity of gemcabene, as PPAR-γ activation is
212 associated with anti-inflammatory activities (25,26) and insulin sensitization (53,54). As
213 expected, the known PPAR-γ activator rosiglitazone showed potent agonist activity (Table 2),
214 while gemcabene did not show any activity up to 100 µM concentration, and modest activation at

300 µM, suggesting that gemcabene possesses little or no direct PPAR-γ activation properties depending upon the species.

In terms of antagonist activities, the data do not indicate a clear antagonist activity of gemcabene against any PPAR subtype (human, mouse and rat), with a clear lack of concentration response.

CONCLUSION

In conclusion, these results rule out efficacious direct agonist or antagonist activities of gemcabene against all three PPAR subtypes: -α, -γ, and -δ, in human, rat and mouse, and infer that gemcabene does not bind to, nor directly activate, the PPAR nuclear hormone receptors to any appreciable extent. These findings are in part in agreement with the significant hypolipidemic efficacy of gemcabene when administered to PPAR-α knockout mice (55,56), suggesting that at least some of gemcabene-mediated lipid regulation is independent of the PPAR-α receptor. The mechanisms of action (MOA) of currently available therapies for hypertriglyceridemia rely on their PPAR activation (56), which incur an inherent safety concern since PPAR-α activation in rodents is associated with specific cancer pathologies (56,57). Gemcabene appears to work through a number of pleiotropic MOAs that we are in the process of elucidating, but the current study shows that gemcabene does not involve a direct PPAR agonist or antagonist component, and that, most likely, in rodents, gemcabene activates PPAR-α responsive genes indirectly.

REFERENCES

1. Kleinman JC, Donahue RP, Harris MI, Finucane FF, Madans JH, Brock DB. Mortality among diabetics in a national sample. Am J Epidemiol 1988;128:389-401.
2. American Heart Association HDass-u. A report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. Circulation 2007;115:e69-e71.
3. Genest JJ, McNamara JR, Salem DN, Schaefer EJ. Prevalence of risk factors in men with premature coronary artery disease. Am J Cardiol 1991;67:1185-9.
4. Williams KJ, Wu X. Imbalanced insulin action in chronic over nutrition: Clinical harm, molecular mechanisms, and a way forward. Atherosclerosis 2016;247:225-82.
5. Srivastava RAK. Dysfunctional HDL in diabetes mellitus and its role in the pathogenesis of cardiovascular disease. Mol Cell Biochem 2017.
6. Frick MH, Elo O, Haapa K et al. Helsinki Heart Study: primary-prevention trial with gemfibrozil in middle-aged men with dyslipidemia. Safety of treatment, changes in risk factors, and incidence of coronary heart disease. N Engl J Med 1987;317:1237-45.
7. Investigators DAIS. Effect of fenofibrate on progression of coronary-artery disease in type 2 diabetes: the Diabetes Atherosclerosis Intervention Study, a randomised study. Lancet 2001;357:905-10.
8. de las Fuentes L, de Simone G, Arnett DK, Davila-Roman VG. Molecular determinants of the cardiometabolic phenotype. Endocr Metab Immune Disord Drug Targets 2010;10:109-23.
9. Chai JT, Choudhury RP. Cardiometabolic interventions - focus on transcriptional regulators. Eur J Cardiovasc Med 2013;2:212-218.
10. Orekhov AN, Mukhamedova N, Ivanova EA, Rizzo M. PPAR in Cardiovascular Disorders. PPAR Res 2016;2016:6293629.
11. Forman BM, Chen J, Evans RM. Hypolipidemic drugs, polyunsaturated fatty acids, and eicosanoids are ligands for peroxisome proliferator-activated receptors alpha and delta. Proc Natl Acad Sci U S A 1997;94:4312-7.
12. Moras D, Gronemeyer H. The nuclear receptor ligand-binding domain: structure and function. Curr Opin Cell Biol 1998;10:384-91.
13. Carson-Jurica MA, Schrader WT, O'Malley BW. Steroid receptor family: structure and functions. Endocr Rev 1990;11:201-20.
14. Schoonjans K, Peinado-Onsurbe J, Lefebvre AM et al. PPARalpha and PPARgamma activators direct a distinct tissue-specific transcriptional response via a PPRE in the lipoprotein lipase gene. EMBO J 1996;15:5336-48.
15. Dressel U, Allen TL, Pippal JB, Rohde PR, Lau P, Muscat GE. The peroxisome proliferator-activated receptor beta/delta agonist, GW501516, regulates the expression of genes involved in lipid catabolism and energy uncoupling in skeletal muscle cells. Mol Endocrinol 2003;17:2477-93.
16. Barroso E, Rodriguez-Calvo R, Serrano-Marco L et al. The PPARbeta/delta activator GW501516 prevents the down-regulation of AMPK caused by a high-fat diet in liver and amplifies the PGC-1alpha-Lipin 1-PPARalpha pathway leading to increased fatty acid oxidation. Endocrinology 2011;152:1848-59.

17. Sprecher DL, Massien C, Pearce G et al. Triglyceride:high-density lipoprotein cholesterol effects in healthy subjects administered a peroxisome proliferator activated receptor delta agonist. Arterioscler Thromb Vasc Biol 2007;27:359-65.
18. Dimopoulos N, Watson M, Green C, Hundal HS. The PPARdelta agonist, GW501516, promotes fatty acid oxidation but has no direct effect on glucose utilisation or insulin sensitivity in rat L6 skeletal muscle cells. FEBS Lett 2007;581:4743-8.
19. Barroso E, Rodriguez-Rodriguez R, Chacon MR et al. PPARbeta/delta ameliorates fructose-induced insulin resistance in adipocytes by preventing Nrf2 activation. Biochim Biophys Acta 2015;1852:1049-58.
20. Srivastava RA, Jahagirdar R, Azhar S, Sharma S, Bisgaier CL. Peroxisome proliferator-activated receptor-alpha selective ligand reduces adiposity, improves insulin sensitivity and inhibits atherosclerosis in LDL receptor-deficient mice. Mol Cell Biochem 2006;285:35-50.
21. Faiola B, Falls JG, Peterson RA et al. PPAR alpha, more than PPAR delta, mediates the hepatic and skeletal muscle alterations induced by the PPAR agonist GW0742. Toxicol Sci 2008;105:384-94.
22. Srivastava RA. Fenofibrate ameliorates diabetic and dyslipidemic profiles in KKAy mice partly via down-regulation of 11beta-HSD1, PEPCK and DGAT2. Comparison of PPARalpha, PPARgamma, and liver x receptor agonists. Eur J Pharmacol 2009;607:258-63.
23. Colville-Nash PR, Qureshi SS, Willis D, Willoughby DA. Inhibition of inducible nitric oxide synthase by peroxisome proliferator-activated receptor agonists: correlation with induction of heme oxygenase 1. J Immunol 1998;161:978-84.
24. Neve BP, Corseaux D, Chinetti G et al. PPARalpha agonists inhibit tissue factor expression in human monocytes and macrophages. Circulation 2001;103:207-12.
25. Murphy GJ, Holder JC. PPAR-gamma agonists: therapeutic role in diabetes, inflammation and cancer. Trends Pharmacol Sci 2000;21:469-74.
26. Moraes LA, Piqueras L, Bishop-Bailey D. Peroxisome proliferator-activated receptors and inflammation. Pharmacol Ther 2006;110:371-85.
27. Cuzzocrea S, Di Paola R, Mazzon E et al. Role of endogenous and exogenous ligands for the peroxisome proliferators activated receptors alpha (PPAR-alpha) in the development of inflammatory bowel disease in mice. Laboratory investigation; a journal of technical methods and pathology 2004;84:1643-54.
28. Staumont-Salle D, Abboud G, Brenuchon C et al. Peroxisome proliferator-activated receptor alpha regulates skin inflammation and humoral response in atopic dermatitis. J Allergy Clin Immunol 2008;121:962-8 e6.
29. Cuzzocrea S, Bruscoli S, Mazzon E et al. Peroxisome proliferator-activated receptor-alpha contributes to the anti-inflammatory activity of glucocorticoids. Mol Pharmacol 2008;73:323-37.
30. Hu Y, Chen Y, Ding L et al. Pathogenic role of diabetes-induced PPAR-alpha down-regulation in microvascular dysfunction. Proc Natl Acad Sci U S A 2013;110:15401-6.
31. Sun Y, Ng L, Lam W et al. Identification and characterization of a novel mouse peroxisome proliferator-activated receptor alpha-regulated and starvation-induced gene, Ppsig. Int J Biochem Cell Biol 2008;40:1775-91.
32. Chakravarthy MV, Pan Z, Zhu Y et al. "New" hepatic fat activates PPARalpha to maintain glucose, lipid, and cholesterol homeostasis. Cell Metab 2005;1:309-22.

33. Chakravarthy MV, Lodhi IJ, Yin L et al. Identification of a physiologically relevant endogenous ligand for PPARalpha in liver. Cell 2009;138:476-88.
34. Sanderson LM, Degenhardt T, Koppen A et al. Peroxisome proliferator-activated receptor beta/delta (PPARbeta/delta) but not PPARalpha serves as a plasma free fatty acid sensor in liver. Mol Cell Biol 2009;29:6257-67.
35. Fernandez-Alvarez A, Alvarez MS, Gonzalez R, Cucarella C, Muntane J, Casado M. Human SREBP1c expression in liver is directly regulated by peroxisome proliferator-activated receptor alpha (PPARalpha). J Biol Chem 2011;286:21466-77.
36. Mandard S, Muller M, Kersten S. Peroxisome proliferator-activated receptor alpha target genes. Cell Mol Life Sci 2004;61:393-416.
37. Shah YM, Morimura K, Yang Q, Tanabe T, Takagi M, Gonzalez FJ. Peroxisome proliferator-activated receptor alpha regulates a microRNA-mediated signaling cascade responsible for hepatocellular proliferation. Mol Cell Biol 2007;27:4238-47.
38. Bisgaier CL, Essenburg AD, Barnett BC et al. A novel compound that elevates high density lipoprotein and activates the peroxisome proliferator activated receptor. J Lipid Res 1998;39:17-30.
39. Bays HE, McKenney JM, Dujovne CA et al. Effectiveness and tolerability of a new lipid-altering agent, gemcabene, in patients with low levels of high-density lipoprotein cholesterol. Am J Cardiol 2003;92:538-43.
40. Stein E, Bays H, Koren M, Bakker-Arkema R, Bisgaier C. Efficacy and safety of gemcabene as add-on to stable statin therapy in hypercholesterolemic patients. J Clin Lipidol 2016;10:1212-22.
41. Clavey V, Copin C, Mariotte MC et al. Cell culture conditions determine apolipoprotein CIII secretion and regulation by fibrates in human hepatoma HepG2 cells. Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology 1999;9:139-49.
42. Liangpunsakul S, Wou SE, Wineinger KD et al. Effects of WY-14,643 on the phosphorylation and activation of AMP-dependent protein kinase. Arch Biochem Biophys 2009;485:10-5.
43. Riserus U, Sprecher D, Johnson T et al. Activation of peroxisome proliferator-activated receptor (PPAR)delta promotes reversal of multiple metabolic abnormalities, reduces oxidative stress, and increases fatty acid oxidation in moderately obese men. Diabetes 2008;57:332-9.
44. Mukherjee R, Sun S, Santomenna L et al. Ligand and coactivator recruitment preferences of peroxisome proliferator activated receptor alpha. J Steroid Biochem Mol Biol 2002;81:217-25.
45. Harrity T, Farrelly D, Tieman A et al. Muraglitazar, a novel dual (alpha/gamma) peroxisome proliferator-activated receptor activator, improves diabetes and other metabolic abnormalities and preserves beta-cell function in db/db mice. Diabetes 2006;55:240-8.
46. Sznaidman ML, Haffner CD, Maloney PR et al. Novel selective small molecule agonists for peroxisome proliferator-activated receptor delta (PPARdelta)--synthesis and biological activity. Bioorg Med Chem Lett 2003;13:1517-21.
47. Bonala S, Lokireddy S, Arigela H et al. Peroxisome proliferator-activated receptor beta/delta induces myogenesis by modulating myostatin activity. J Biol Chem 2012;287:12935-51.
48. Quintela AM, Jimenez R, Piqueras L et al. PPARbeta activation restores the high glucose-induced impairment of insulin signalling in endothelial cells. Br J Pharmacol 2014;171:3089-102.

49. Bojic LA, Telford DE, Fullerton MD et al. PPARdelta activation attenuates hepatic steatosis in Ldlr-/- mice by enhanced fat oxidation, reduced lipogenesis, and improved insulin sensitivity. J Lipid Res 2014;55:1254-66.
50. Olson EJ, Pearce GL, Jones NP, Sprecher DL. Lipid effects of peroxisome proliferator-activated receptor-delta agonist GW501516 in subjects with low high-density lipoprotein cholesterol: characteristics of metabolic syndrome. Arterioscler Thromb Vasc Biol 2012;32:2289-94.
51. Lee G, Elwood F, McNally J et al. T0070907, a selective ligand for peroxisome proliferator-activated receptor gamma, functions as an antagonist of biochemical and cellular activities. J Biol Chem 2002;277:19649-57.
52. Seimandi M, Lemaire G, Pillon A et al. Differential responses of PPARalpha, PPARdelta, and PPARgamma reporter cell lines to selective PPAR synthetic ligands. Anal Biochem 2005;344:8-15.
53. Bisgaier CL, Newton RS. Statin-Carboxyalkylether Combinations. United States: Warner-Lambert Company, LLC, New York, NY (US), 2009.
54. Bakker-Arkema R, Bisgaier C. Effect of Gemcabene on Insulin Sensitivity in Nondiabetic, Obese Subjects. ACC 2017, Washington D.C., 2017.
55. Bisgaier C, Srivastava RA. Novel New Chemical Entity, Gemcabene, Shows Significant Lipid Regulation Independent of PPAR-α. . Circulation AHA Abstracts 2015., 2015.
56. Peters JM, Hennuyer N, Staels B et al. Alterations in lipoprotein metabolism in peroxisome proliferator-activated receptor alpha-deficient mice. J Biol Chem 1997;272:27307-12.
57. Cheung C, Gonzalez FJ. Humanized mouse lines and their application for prediction of human drug metabolism and toxicological risk assessment. J Pharmacol Exp Ther 2008;327:288-99.
58. Lehmann JM, Lenhard JM, Oliver BB, Ringold GM, Kliewer SA. Peroxisome proliferator-activated receptors alpha and gamma are activated by indomethacin and other non-steroidal anti-inflammatory drugs. J Biol Chem 1997;272:3406-10.

TABLE LEGENDS

Table 1: Descriptive information on Gemcabene and Reference Agents: Type, Chemical Abstract Services (CAS) Number, Molecular Weights (MW) and Molecular Formulae (MF)

Table 2: PPAR Transcriptional Activation by Gemcabene and Reference Compounds (or Commercial Drugs) in Cell-Based Transactivation Assays Using Gal-4 PPAR Chimeras Receptors

FIGURE LEGENDS

Figure 1: Structures of gemcabene and reference compounds evaluated in the PPAR transactivation assays

Figure 2: Transactivation Methodology, for the agonist (panel A) and antagonist (panel B) assays. Assays were carried out in 96 well plates using a proprietary cell line from Indigo Biosciences, State College, PA, 16801, USA. This proprietary cell line expresses a hybrid receptor comprising the N-terminal Gal4 DNA-binding domain fused to the ligand-binding domain (LBD) of the specific nuclear receptor. The reporter gene used in this assay is the firefly luciferase, which is functionally linked to the Gal4 upstream activation sequence. The fluorescence-based Live Cell Multiplex Assay and luminescence-based nuclear receptor assay were performed sequentially using the same assay wells. For the agonist assay, Nuclear Receptor reporter cells were incubated with the test compound for 24 h followed by the Live Cell Multiplex Assay as shown and Relative Fluorescence was read. Media were removed by aspiration, the detection reagent was added and relative Luciferase activity was measured in a luminometer. The antagonist assay was performed in the same manner except in the presence of agonist at a concentration at 2x that of the EC_{80}.

NR, nuclear receptor; LCMA, Live Cell Multiplex Assay; RFU, Relative Fluorescence Unit; RLU, Relative Luciferase Unit

Figure 3: Graphical presentation of the transactivation assays for PPAR-α, PPAR-δ, and PPAR-γ agonists using GAL4-LBD. Luciferase activity was measured following treatments with the indicated reference agents or test agent, gemcabene. Strong (GW590735, muraglitazar), mild (WY-14643, fenofibric acid), and weak (gemfibrozil) PPAR-α agonists were used as reference agents in this assay against human, rat, and mouse PPAR-α as indicated. For PPAR-δ, strong (GW0742, GW501516, L165041) PPAR-δ agonists were used as reference agents in this assay against human, rat and mouse PPAR-δ as shown. Similarly, strong PPAR-γ agonists were used as reference agents in this assay against human, rat, and mouse PPAR-γ as indicated in the figure. Note that there is a single graph for mouse and rat PPAR-γ assay, as only the mouse transactivation assays was performed, since the DNA binding domain between the mouse and rat receptors are homologous.

Figure 4: Graphical presentations of the transactivation assay for PPAR antagonist activity. Luciferase activity was measured following treatments with the indicated test agent, gemcabene, against human, rat, and mouse PPAR-α, PPAR-δ and PPAR-γ in the presence of appropriate agonists (WY-1453 for PPAR-α, GW501516 for PPAR-δ, and Rosiglitazone for PPAR-γ). While we could not find a reference agent for PPAR-α antagonist, we used GSK3787 (58) and T0070907 (51) as PPAR- δ and PPAR-γ antagonists, respectively. Note that there is a single graph for mouse and rat PPAR-γ assay, as only the mouse transactivation assays was performed, since the DNA binding domain between the mouse and rat receptors are homologous.

Table 1: Descriptive Information on Gemcabene and Reference Agents: Type, Chemical Abstract Service (CAS) Number, Molecular Weights (MW) and Molecular Formulae (MF)

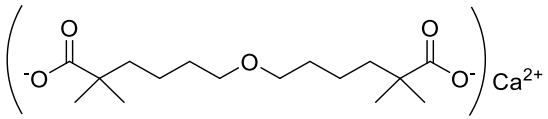
Assay Class	Compound	IC_{50}/EC_{50} (μM) (literature)	CAS No.	MW	MF
PPAR-α	**Gemcabene**		183293-82-5	302.05	C16H30O5
PPAR-α	Gemfibrozil	193 (44)	25812-30-0	250.33	$C_{15}H_{22}O_3$
PPAR-α	Fenofibric acid	18-30 (44)	42017-89-0	318.75	C17H15ClO4
PPAR-α	GW590735	0.004 (43)	622402-22-6	478.5	$C_{23}H_{21}F_3N_2O_4S$
PPAR-α	WY-14643	6.4 (41)	50892-23-4	323.8	$C_{14}H_{14}ClN_3O_2S$
PPAR-α	Muraglitazar	0.34 (45)	317318-84-6	471.49	$C_{21}H_{17}F_4NO_3S_2$
PPAR-δ	**Gemcabene**		183293-82-5	302.05	$C_{16}H_{30}O_5$
PPAR-δ	Eicosapentaenoic acid (EPA)	Unknown	10417-94-4	302.45	$C_{20}H_{30}O_2$
PPAR-δ	GW0742	0.0011(46)	317318-84-6	471.49	$C_{21}H_{17}F_4NO_3S_2$
PPAR-δ	GW501516	0.0011 (49)	317318-70-0	453.5	$C_{21}H_{18}F_3NO_3S_2$
PPAR-δ	L165041	0.05 (47)	79558-09-1	402.4	$C_{22}H_{26}O_7$
PPAR-γ	**Gemcabene**		183293-82-5	302.05	$C_{16}H_{30}O_5$
PPAR-γ	Rosiglitazone (BRL49653)	0.006 - 0.043 (45)	122320-73-4	357.43	$C_{18}H_{19}N_3O_3S$
PPAR-γ	Indomethacin	40 (58)	53-86-1	357.8	$C_{19}H_{16}ClNO_4$
PPAR-γ	Muraglitazar	0.004 (45)	317318-84-6	471.49	$C_{21}H_{17}F_4NO_3S_2$

Table -2 PPAR Transcriptional Activation by Gemcabene and Reference Compounds (or Commercial Drugs) in Cell-Based Transactivation Assays Using Gal-4 PPAR Chimeras Receptors

Assay Class	Compound	Concentration Range Tested (nM)	Fold Change[1]			EC_{50} (µM)			Comments
			Human	Mouse	Rat	Human	Mouse	Rat	
PPAR-α	**Gemcabene**	137-300,000	1.0-5.2	1.0-2.0	1.0-1.7	ND[2]	NA[3]	NA[3]	No concentration-response
	Fenofibric acid	137-300,000	2.4-30	1.3-61	0.66-97	47.1	35.8	29.6	
	Gemfibrozil	137-300,000	2.7-18	1.2-41	1.1-51	201	111.53	122.3	
	GW590735	0.457-1,000	3.1-21	1.4-89	0.86-83	2.30E-02	0.098	2.2	
	WY-14643	45.7-100,000	2.3-25	1.2-84	0.98-145	32.6	18.6	10.8	
	Muraglitazar	4.57-10,000	2.5-19	0.95-1.3	1.0-1.3	6.5	NA[3]	NA[3]	
PPAR-δ	**Gemcabene**	137-300,000	1.0-3.2	1.0-1.1	1.0-1.4	ND[2]	NA[3]	NA[3]	No Activity
	EPA	137-300,000	1.7-75	1.1-11	1.1-13	90.5	ND[2]	ND[2]	Cell Death at 300 µM
	GW0742	0.0457-100	3.4-409	1.1-188	1.1-253	1.90E-03	0.036	0.059	
	GW501516	0.0457-100	2.0-383	1.1-165	1.10-218	1.60E-03	0.044	63	
	L165041	0.457-1,000	1.7-315	1.0-112	1.0-37	1.70E-02	0.63	ND[2]	
PPAR-γ	**Gemcabene**	137-300,000	1.0-5	1.0-3.6[3]		ND[2]	ND[2]		No concentration-response
	Rosiglitazone	0.0457-100	0.92-35	1.1-76[3]		1.49E-01	0.161		
	Indomethacin	137-300,000	1.1-52	1.0-90[3]		11.92	12.14		Cell Death at 300 µM
	Muraglitazar	0.0457-100	1.2-16	0.89-14[3]		2.17E-01	ND[2]		

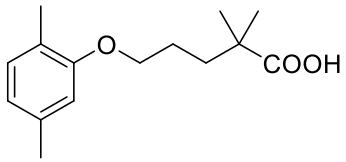
[1]Fold change indicates minimal and maximal activation;
[2]EC_{50} could not be determined;
[3]For PPAR gamma, only the mouse transactivation assay was performed as the DNA binding domain between the mouse and rat receptors are homologous

Figure 1

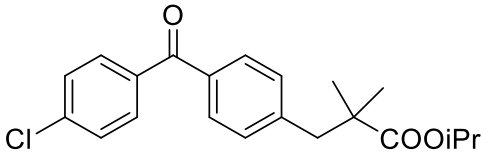


Gemcabene Calcium –

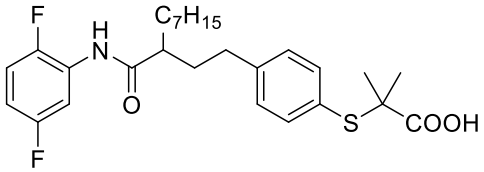
(6,6'-oxybis (2,2-dimethylhexanoic acid) monocalcium salt



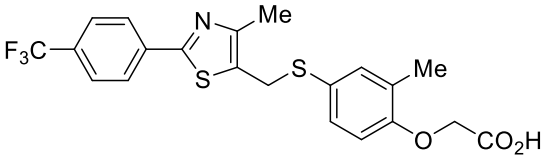
Gemfibrozil – PPAR-α activator



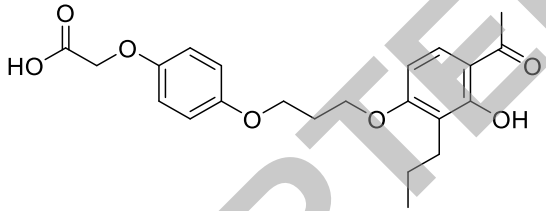
Fenofibrate – PPAR-α activator



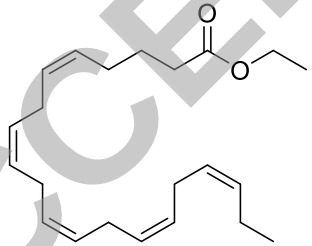
GW-9578 – PPAR-α activator



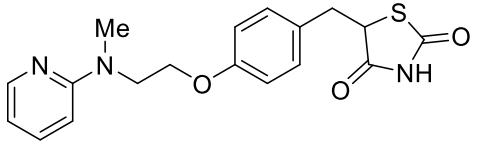
GW501516 – PPAR-δ activator



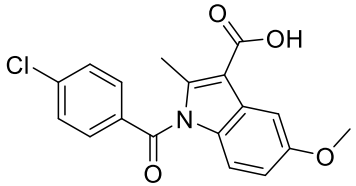
L-165041 – PPAR-δ activator



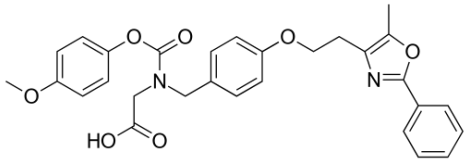
EPA – PPAR-δ activator



Rosiglitazone – PPAR-γ activator



Indomethacin – PPAR-γ activator



Muriglitazar - PPAR-γ activator

Figure 2

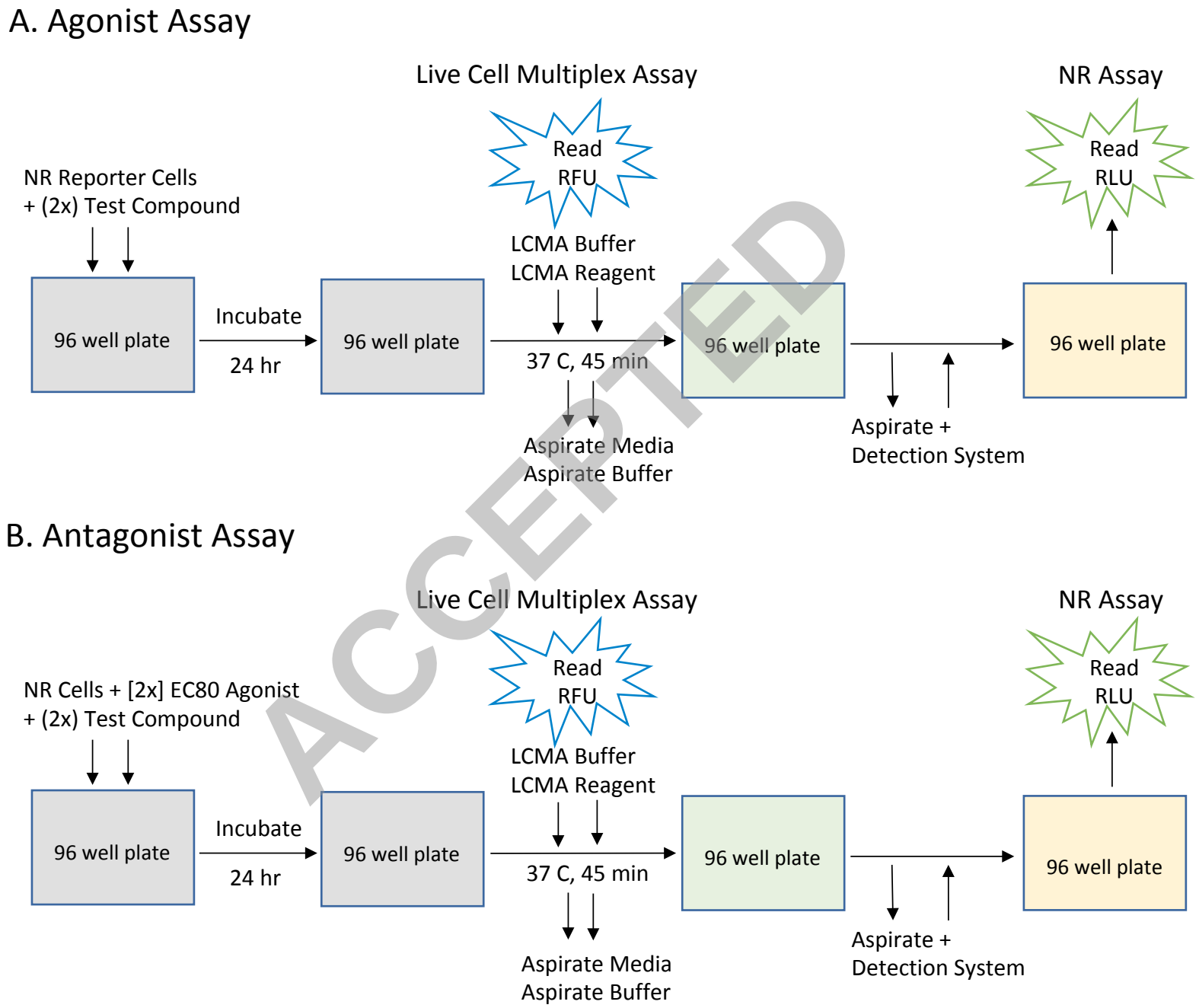


Figure 3

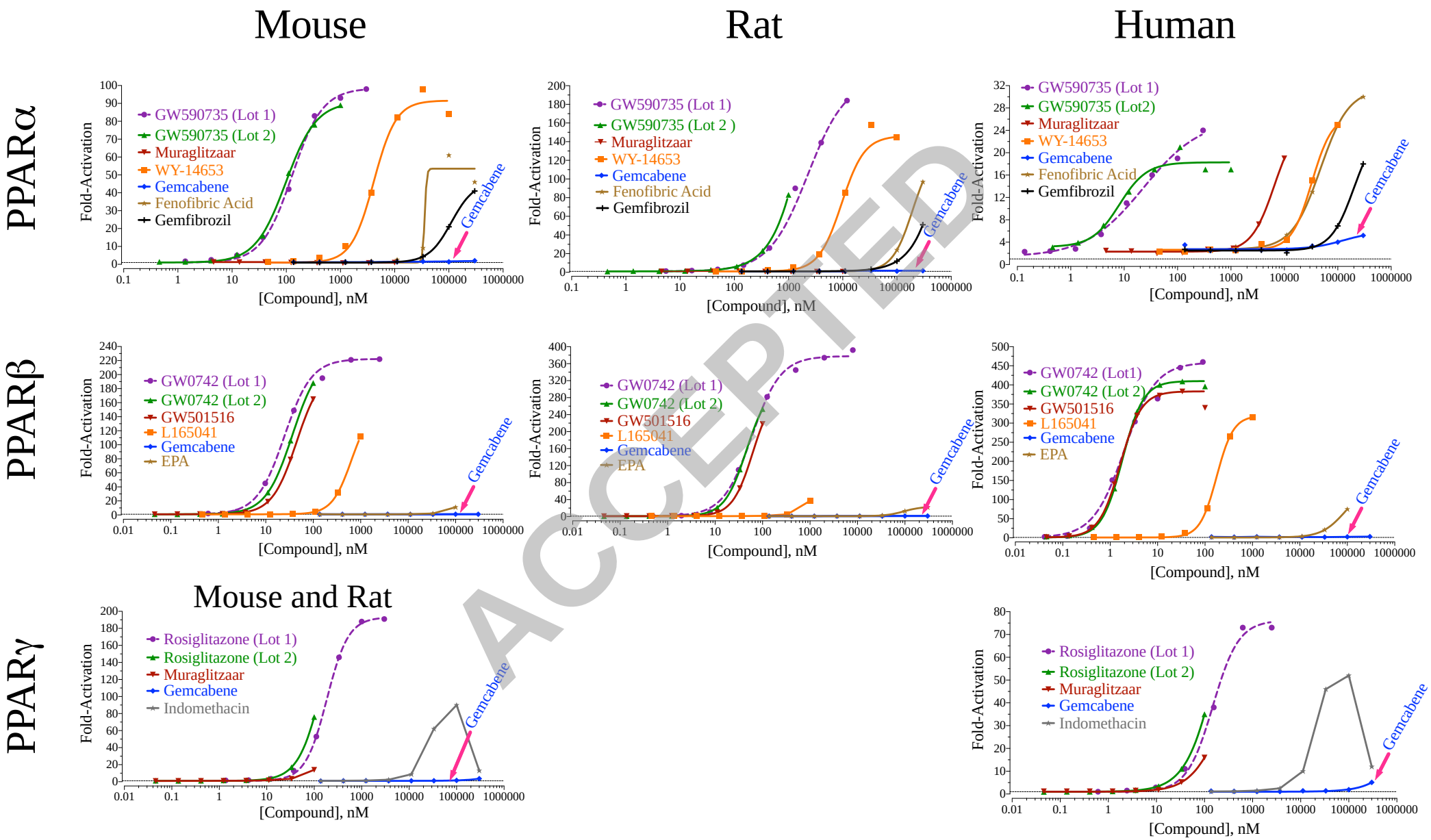


Figure 4