Identification of peroxisome proliferator-activated receptor ligands from a biased chemical library

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Background: The peroxisome proliferator-activated receptors (PPARs) were cloned as orphan members of the nuclear receptor superfamily of transcription factors. The identification of subtype-selective ligands for PPARα and PPARγ has led to the discovery of their roles in the regulation of lipid metabolism and glucose homeostasis. No subtype-selective PPARδ ligands are available and the function of this subtype is currently unknown.

Results: A three-component library was designed in which one of the monomers was biased towards the PPARs and the other two monomers were chosen to add chemical diversity. Synthesis and screening of the library resulted in the identification of pools with activity on each of the PPAR subtypes. Deconvolution of the pools with the highest activity on PPARδ led to the identification of GW 2433 as the first high-affinity PPARδ ligand. [^{3}H]GW 2433 is an effective radioligand for use in PPARδ competition-binding assays.

Conclusions: The synthesis of biased chemical libraries is an efficient approach to the identification of lead molecules for members of sequence-related receptor families. This approach is well suited to the discovery of small-molecule ligands for orphan receptors.

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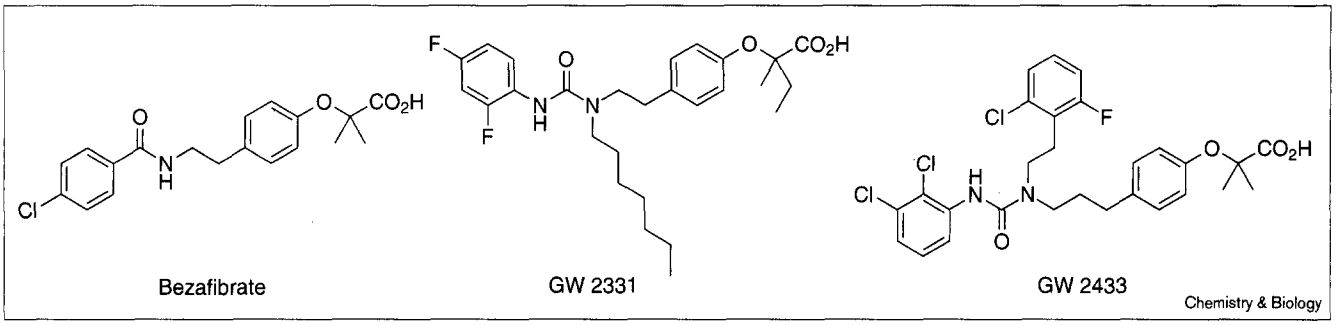
Introduction

The peroxisome proliferator-activated receptors (PPARs) are members of the superfamily of ligand-activated transcription factors that includes the receptors for the steroid hormones, retinoids, thyroid hormone and vitamin D [1]. The PPARs were originally cloned as orphan receptors for which the cognate hormones were unknown. Three receptor subtypes have been identified in humans and rodents, which are known as PPARα, PPARγ, and PPARδ. In addition, three subtypes identified in *Xenopus laevis* have been designated PPARα, PPARγ, and PPARβ. Although the PPARα and PPARγ subtypes appear to be homologous across species, it is currently unclear whether the PPARδ and PPARβ subtypes are functional homologs or two independent receptors [2]. Like other members of the nuclear receptor family, the PPARs contain a DNA-binding domain (DBD) and a carboxy-terminal ligand-binding domain (LBD). The PPARs show > 85% sequence homology in the DBDs and > 60% sequence homology in the LBDs.

Evidence that the PPARs may have different physiological functions was suggested from studying their tissue expression patterns. The PPARα subtype is expressed predominantly in liver, whereas PPARγ is expressed predominantly in fat, and PPARδ is widely expressed in many tissues [3]. Following the identification of synthetic subtype-selective ligands for the PPARα and PPARγ subtypes [4], pharmacological studies have established the role of PPARα in the regulation of hepatic lipid metabolism and the role of PPARγ in the regulation of adipocyte function and glucose homeostasis [5,6]. Notably, the fibrate family of lipid-lowering drugs and the thiazolidinedione family of anti-diabetic drugs function through binding to these two PPAR subtypes [7]. In addition, recent data suggest that PPARα and PPARγ may be hormone receptors for naturally occurring unsaturated fatty acids and certain eicosanoid metabolites [8–10]. Together, these studies suggest that one function of the PPARα and PPARγ subtypes is to coordinate the body's physiological response to dietary fatty acids through the regulation of lipid storage and catabolism [2].

In contrast to the progress in elucidating the function of PPARα and PPARγ, the physiological role of PPARδ is

Figure 1



The structures of fibrates described in this study.

currently unknown. Although PPARδ also responds to fatty acids in cell-based reporter gene assays [3,11,12], the lack of synthetic PPARδ ligands has precluded detailed pharmacological study of this subtype. Thus, PPARδ remains an orphan receptor of unknown function. The identification of synthetic PPARδ ligands will allow pharmacological evaluation of the function of this subtype and may provide new opportunities for drug discovery.

The technology of combinatorial chemistry is being widely employed to generate large diverse compound libraries for use in high-throughput screening for new lead generation [13,14]. Using similar chemical techniques, smaller libraries with lower compound diversity (so-called focused libraries) are often used to optimize the initial leads for potency and selectivity against a particular target [15]. We reasoned that, for orphan targets that belong to sequence-related receptor superfamilies, the screening of compound libraries biased toward the appropriate target class would be an efficient approach to lead generation. These libraries would be designed to have a compound diversity lower than a large combinatorial library but greater than a small focused library. We set out to test this concept through the solid phase synthesis (SPS) of biased compound libraries for the PPARs. Our goal was to identify ligands with various subtype selectivities to support additional pharmacological investigation of receptor function. We have designed and synthesized a library of 480 trisubstituted urea analogs that incorporated a phenoxyisobutyric acid monomer, commonly found in the fibrate class of lipid-lowering drugs (eg., bezafibrate, Figure 1) [16]. This biased library showed activity on all three PPAR subtypes and allowed identification of GW 2433 as the first high-affinity PPARδ ligand.

Results

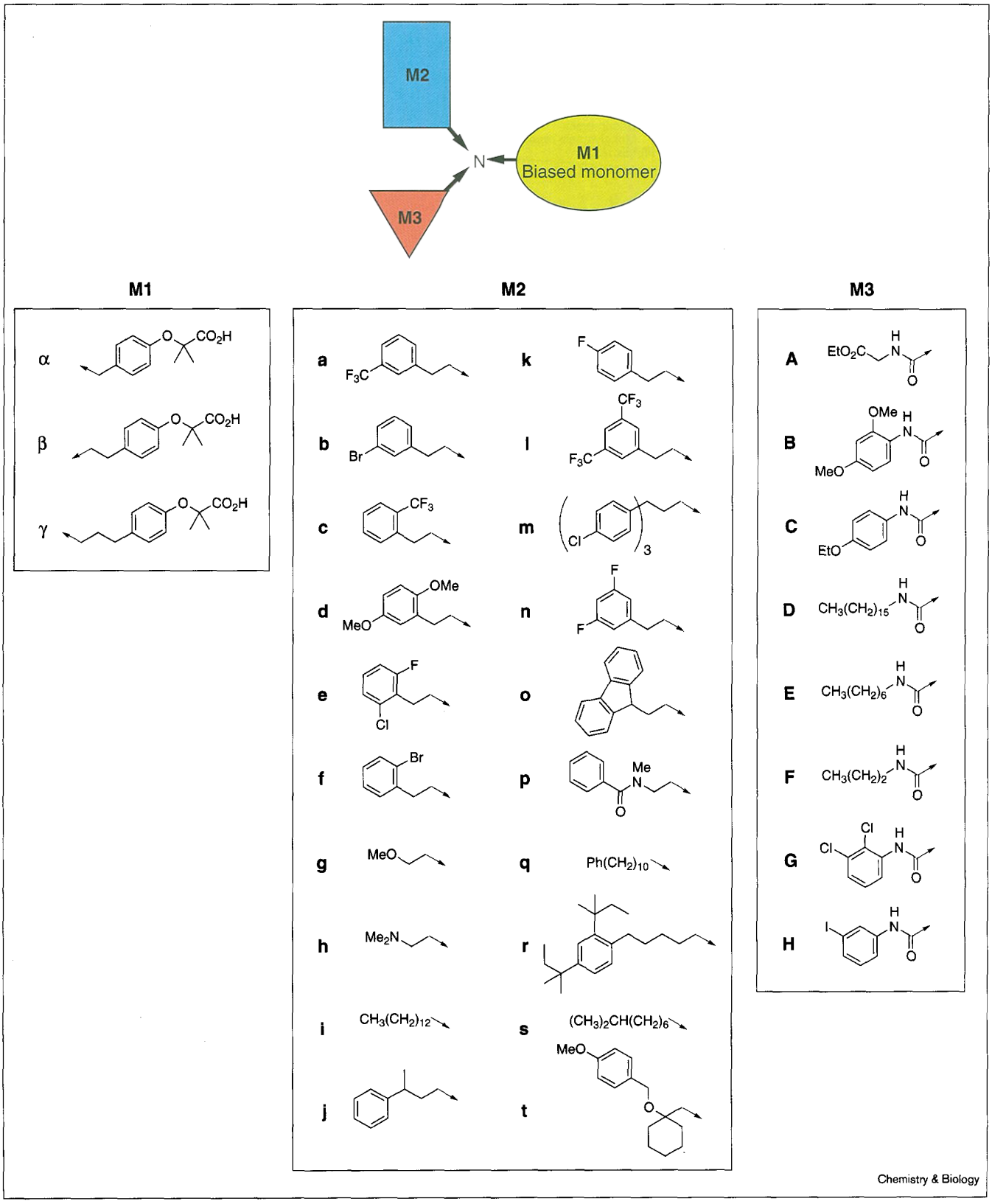
Design and synthesis of the fibrate library

The strategy for the construction of our library was based on the premise that the phenoxyisobutyric acid group found in the fibrate class of lipid-lowering drugs [16] would be a biasing monomer toward the PPAR receptors. For example, we have shown that the fibrate GW 2331 (Figure 1), containing a lipophilic trisubstituted urea, is a high-affinity ligand for PPARα and PPARγ [8]. In addition, GW 2331 was a potent activator of xPPARβ (*Xenopus*), although it activated human and mouse PPARδ only at doses >1 μM [8]. Thus, we chose to design a library that added two points of diversity to the urea while maintaining the fibrate portion of the molecule (Figure 2).

We have recently developed an SPS of fibrate analogs from the combination of an amino-substituted fibrate, a carboxylic acid and an isocyanate [17]. For the current library, three fibrate monomers (M1, Figure 2) were synthesized that contained 1, 2 or 3 methylenes between the phenyl ring and terminal amino group (**1–3**, Figure 3). The selection of the carboxylic acid (M2, Figure 2) and isocyanate monomers (M3, Figure 2) was driven by our desire to explore the steric and electronic requirements for PPAR activity as well as to identify compounds with novel selectivities. Starting from all commercially available carboxylic acids and isocyanates, the list of potential monomers was pared down by a series of logical steps. First, all of the monomers with molecular weight >300 or functionality incompatible with the solid phase chemistry were removed. Monomers with a large number of rotatable bonds were also eliminated. The remaining monomers were incorporated into a series of three-dimensional virtual libraries to calculate the electrostatic potential and molecular surface of each theoretical product. Finally, a Spacefill experimental design [18] was used to select the 20 carboxylic acids (M2) and eight isocyanates (M3) that gave the most diverse library within the constraints of our design strategy (Figure 2).

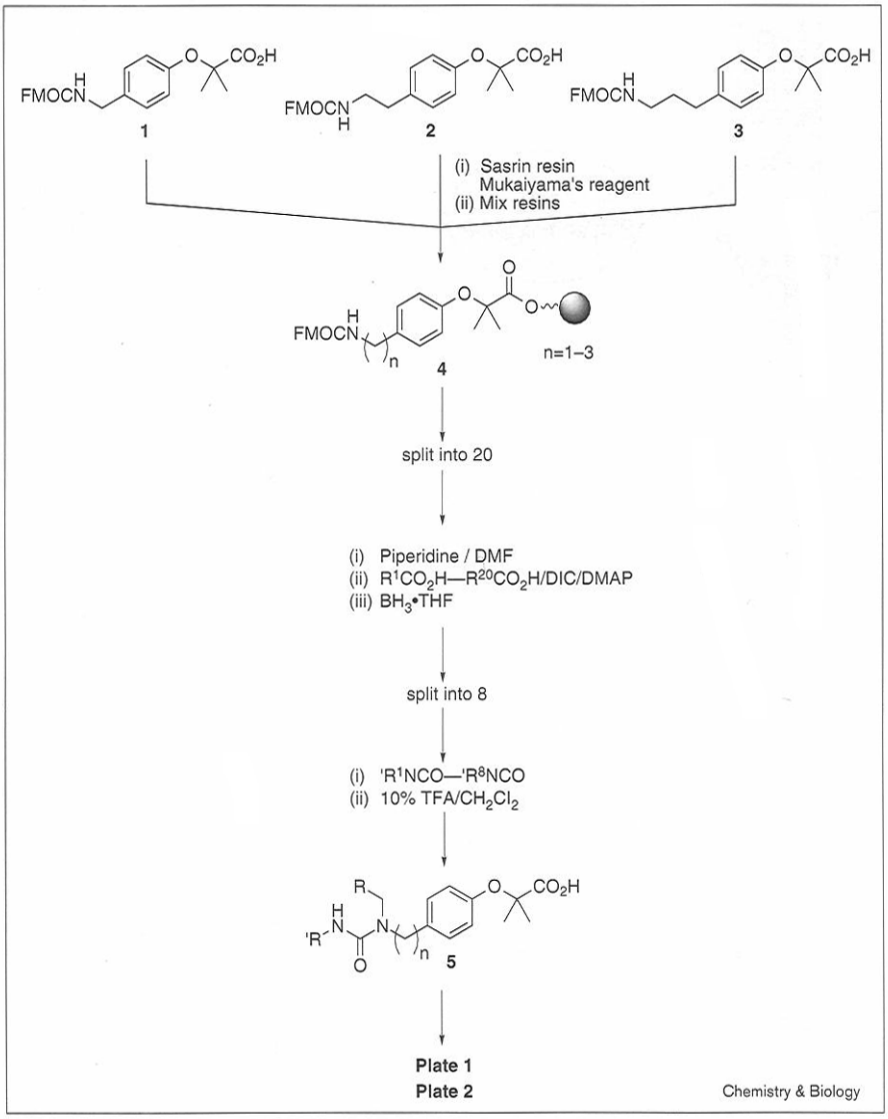
Each of the fibrate monomers (**1–3**) was loaded onto Sasrin resin and quantified by 9-fluorenylmethoxycarbonyl (FMOC) analysis (Figure 3). We chose to synthesize the library by pooling the resin-bound fibrate monomers and thus shorten the synthesis and screening

Figure 2



The design of the biased library. M1 is the biasing fibrate monomer. M2 and M3, derived from carboxylic acids and isocyanates, were chosen for diversity by experimental design.

Figure 3



Solid phase synthesis of the fibrate library [17]. DMF, dimethyl formamide; TFA, trifluoroacetic acid; THF, tetrahydrofuran; DIC, 1,3-diisopropylcarbodiimide; DMAP, 4-dimethylaminopyridine.

time. The resins (**4**) were pooled in equimolar quantities and then split into 20 aliquots. Each aliquot was deprotected and then coupled with one of the 20 carboxylic acids. Following borane reduction, each aliquot was split into eight and reacted with the isocyanates. The final library of 480 fibrates (**5**) was synthesized in 8 × 10 parallel arrays in two microtiter plates. Each well contained a pool of three compounds resulting from the three fibrate monomers (M1) with a unique combination of a carboxylic acid (M2) and an isocyanate (M3; Figure 4a).

PPAR activation profile of the fibrate library

The fibrate library was screened at a concentration of 5 μM in cell-based reporter gene assays against each of the three human PPAR subtypes. We chose to employ PPAR–GAL4 chimeric receptors since this assay format eliminated the background activity from endogenous receptors and allowed quantitation of relative activity across the three PPAR subtypes using the same reporter gene [19]. The activation data was expressed as the percentage activation relative to 10 μM GW 2331, which showed 60-, 25- and 12-fold activation of PPARα, PPARγ and PPARδ, respectively (data not shown). Notably, the library showed different activity profiles on the three PPAR subtypes (Figure 4b). The data revealed that PPARα was the most promiscuous of the three subtypes with more than half of the wells showing significant activity (>25% of control). By contrast approximately one third of the wells were active on PPARγ and less than one fifth were active on PPARδ. The patterns of activity showed that aromatic M3 substituents (**B**, **C**, **G**, **H** in Figure 2) were active on all three subtypes, but that aliphatic M3 substituents (**A**, **E**, **F** ;Figure 2) showed more sporadic activity especially on PPARγ and PPARδ. Among the M2

Figure 4

The fibrate library. **(a)** Array format of the fibrate library. Monomers M2 and M3 are shown on the axes of the plates. **(b)** Activation of human PPAR–GAL4 chimeras by the fibrate library. Data were expressed as percentage activation compared to 10 μM GW 2331 [8].

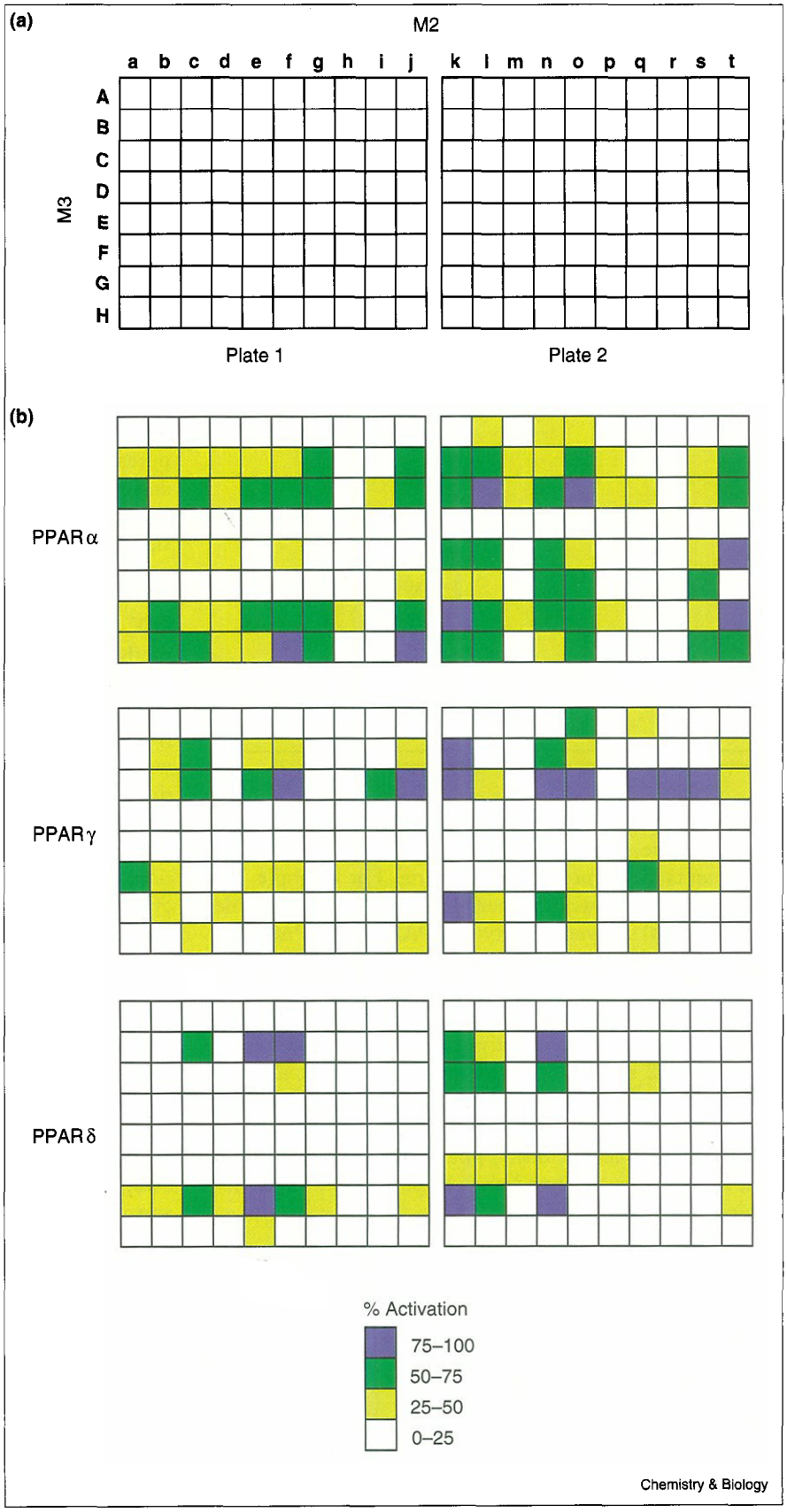


Table 1

PPARδ activity of the deconvoluted pools.

Compound*	EC_{50} (nM)
αeB	>10,000
βeB	>10,000
γeB	1900
αeG	>10,000
βeG	>10,000
γeG (GW 2433)	160
αfB	>10,000
βfB	6300
γfB	6300
αkG	>10,000
βkG	2000
γkG	250
αnB	>10,000
βnB	>10,000
γnB	7900
αnG	>10,000
βnG	>10,000
γnG	1600

*Structures can be constructed from the monomers in Figure 2.

substituents, certain phenethyl groups (**e**, **f**, **k**, **l**, **n**; Figure 2) were active on all three subtypes, whereas other M2 substituents were more discriminating. For example M2 substituent (**t**) showed primarily PPARα activity, while substituent (**o**) showed activity on PPARα and PPARγ but not PPARδ. Certain combinations of M2 and M3 substituents gave wells that showed predominant activity on a single subtype at the dose tested. Thus, **tE** was selective for PPARα, and **rC** was selective for PPARγ. Other combinations of M2 and M3 substituents, such as **kG**, showed activity on all three PPAR subtypes. All of the pools with the highest PPARδ activity (**eB**, **eG**, **fB**, **kG**, **nB**, **nG** in Figure 4) contained modified phenethyl M2 substituents (**e**, **f**, **k**, **n** in Figure 2) and showed some cross-reactivity with either PPARα or PPARγ.

From this initial analysis, the binding pockets of the PPAR subtypes appear to be grossly similar, but with subtle differences that can be exploited to identify subtype-selective ligands. Thus, PPARα is able to tolerate aliphatic M3 substituents (for example **E**) better than PPARγ or PPARδ, whereas only PPARγ will tolerate large long-chain aliphatic M2 substituents (for example **q** or **r**, Figure 2). The PPARδ pocket may be the least tolerant of different substituents since it showed the fewest active wells in the library.

Identification of a high-affinity PPARδ ligand

In part, our need to pool the fibrate monomers was dictated by the limitation in the throughput of the cell-based screening assay [20]. We have recently developed radioligands for the PPARα [8] and PPARγ [19] subtypes that allow rapid screening of compound libraries by standard competition-binding assays. The development of a comparable tool for the PPARδ subtype would permit future libraries to be synthesized and screened as discrete compounds against all three receptors in parallel. For this reason we identified the six most active pools (Figure 4b; **eB**, **eG**, **fB**, **kG**, **nB**, **nG**) and synthesized the individual components (M1; **α**, **β**, **γ**) of each pool by SPS. The resulting 18 compounds (Table 1) were assayed for activity on PPARδ with the generation of full concentration–response curves. Only compounds possessing the fibrate monomer with the three methylene linker (**γ**) were found to be potent activators of PPARδ. Compounds **γeG** (GW 2433) and **γkG** were the most potent with EC_{50} values of 160 nM and 250 nM, respectively. Both compounds also showed significant activity on PPARα (EC_{50} ~300–500 nM) but were inactive on PPARγ (data not shown).

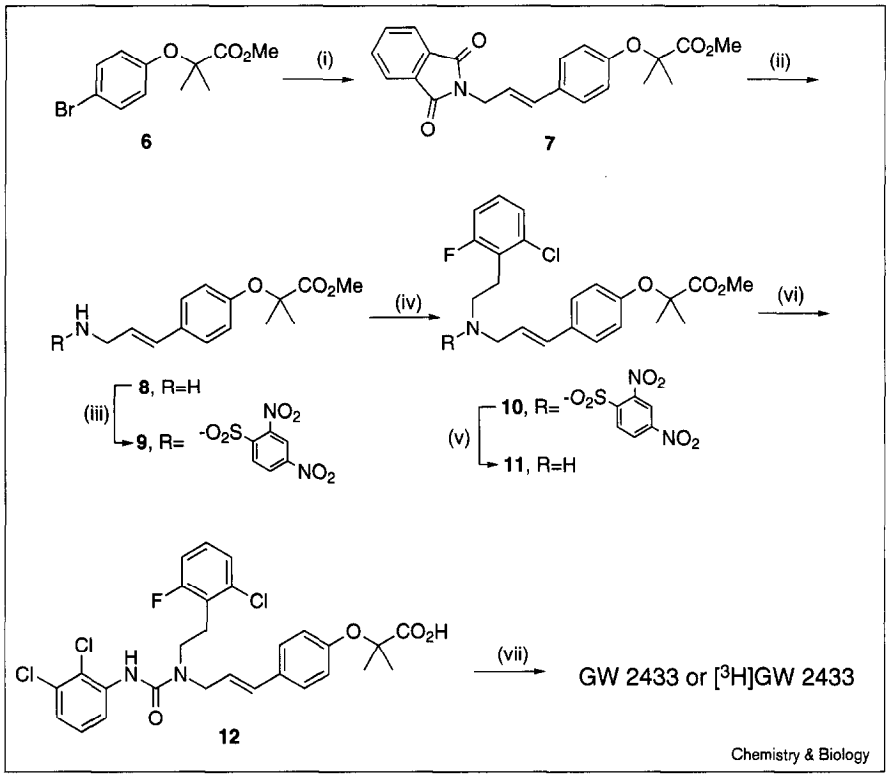
GW 2433 (**γeG**) was resynthesized by a solution phase method that allowed incorporation of radioactivity by tritiation of an unsaturated derivative (Figure 5). The bromoester (**6**) was subjected to a Heck reaction [21] with *N*-allylphthalimide, and the resulting product (**7**, Figure 5) was deprotected with hydrazine. The amine (**8**) was derivatized as the 2,4-dinitrosulfonamide (**9**), which was condensed with 2-chloro-6-fluorophenylethanol by the method of Fukuyama *et al.* [22]. Removal of the sulfonamide, reaction of the free amine (**11**) with 2,3-dichlorophenylisocyanate and saponification of the ester gave the unsaturated ureido-fibrate (**12**). Catalytic hydrogenation of **12** proceeded smoothly to give GW 2433. Reduction of **12** with tritium gas gave [^{3}H]GW 2433 with a specific activity of 38 Ci/mmol. [^{3}H]GW 2433 showed specific saturable binding to bacterially expressed PPARδ (Figure 6a). A nonlinear least squares fit of the data gave a K_d of 13 nM. In competition-binding assays GW 2433 bound to PPARδ with an apparent K_i of 40 nM, a value similar to the K_d determined by saturation binding (Figure 6b). GW 2433 also competed for [^{3}H]GW 2331 binding to PPARα with an apparent K_i of 120 nM, but had no effect on [^{3}H]BRL 49653 binding to PPARγ at doses up to 10 μM.

Discussion

The advent of the technology of combinatorial chemistry has provided new opportunities for the discovery of receptor ligands. The split/mix SPS method has been used to generate large chemical libraries of high diversity for screening against a wide range of targets [14,15]. As an alternative to the random approach, we have chosen to use SPS technology to generate compound libraries using a design methodology that biases the library toward families

Figure 5

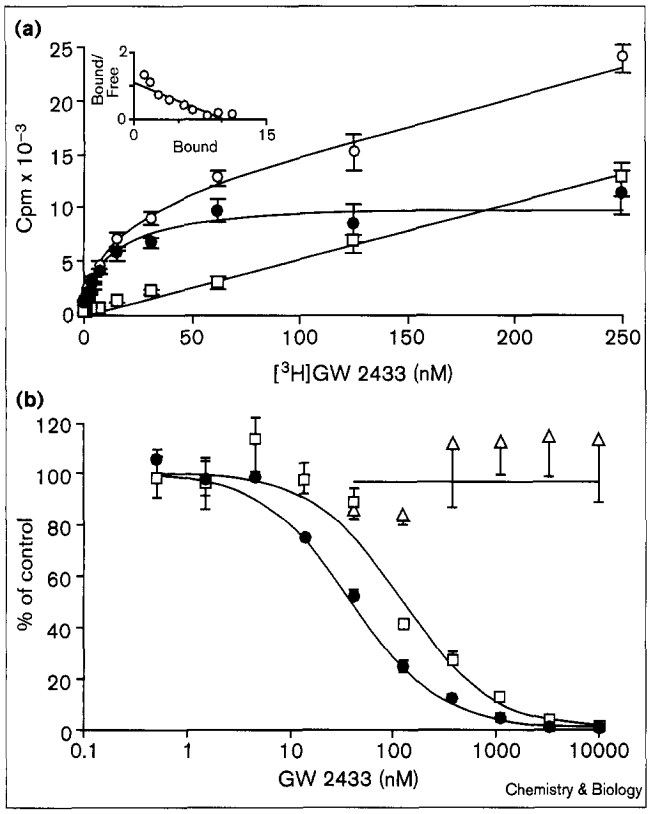
Solution synthesis of GW 2433. (i) $Pd(OAc)_2$, $P(o\text{-}Tol)_3$, MeCN, *N*-allylphthalimide; yield 66%. (ii) NH_2NH_2; yield 75%. (iii) 2,4-Dinitrophenylsulfonylchloride, pyridine; yield 45%. (iv) DEAD, PPh_3, 2-chloro-6-fluorophenylethanol; yield 72%. (v) $HSCH_2CO_2H$, Et_3N; yield 78%. (vi) 2,3-Dichlorophenylisocyanate; then NaOH; yield 48%. (vii) H_2, 10% Pd-C; or T_2,10% Pd-C; yield 93%.



of sequence-related receptors. This approach combines the insights of traditional medicinal chemistry with the benefits of rapid synthesis using solid phase chemistry. In the current example, since fibrates were known to have activity on multiple PPARs [8–10], phenoxyisobutyric acid was chosen as the biasing monomer that was present in each of the 480 members of the library. The screening results showed that this strategy led to the generation of a compound library in which 20–50% of the wells showed activity on the different PPAR subtypes. By synthesizing the library in a parallel array format, the resulting patterns of activity contained information about the preferred monomers that led to subtype selectivity. Thus, we were able to rapidly identify those monomers which gave interesting PPAR selectivity profiles. For example, when M2 was derived from 1-(4-methoxybenzyloxy)cyclohexane carboxylic acid (**t**) the resulting pools showed primarily PPARα activity. When M2 was derived from 10-phenyldecanoic acid (**q**), however, the resulting pools showed primarily PPARγ activity. In addition, unique combinations of M2 and M3 substituents that gave receptor selectivity were easily identified; **tE** was PPARα selective, and **rC** was PPARγ selective. We chose not to deconvolute these pools, however, as we had already identified potent ligands for these two PPAR subtypes [8,19]. Instead we opted to deconvolute the six pools most active on PPARδ. Resynthesis of the 18 individual compounds led to the identification of GW 2433 (**γeG**) as the most potent compound on PPARδ, although it also showed significant PPARα activity. Thus, the fibrate monomer with the 3-methylene linker appears to be permissive to PPARα and PPARδ, but excludes PPARγ activity. Identification of PPARδ subtype selective compounds devoid of PPARα activity will require synthesis of new focused libraries to optimize the current leads.

The activation profiles of the fibrate library demonstrated that PPARα is the most promiscuous of the subtypes, with PPARγ and PPARδ showing more restricted patterns of activity. Historically, the fibrates were discovered through systematic evaluation of branched-chain fatty acids for their ability to regulate lipid metabolism in animals [23]. Therefore, it is interesting to note that recent evidence suggests that the PPARs function as hormone receptors for unsaturated fatty acids and their eicosanoid metabolites [8–10]. PPARα is expressed predominantly in the liver, where it regulates many of the genes involved in the catabolism of fatty acids. The PPARα subtype may have evolved to respond to the wide range of naturally occurring fatty acids that are processed by the liver [24]. Notably, data generated with cell-based reporter gene assays showed that different classes of fatty acids activate PPARα, irrespective of chain length, degree of unsaturation or oxidation [25,26]. In comparison to PPARα, our data show that PPARδ is activated by a more restricted range of synthetic compounds, which suggests that it may

Figure 6



(a) Saturation binding of [^{3}H]GW 2433 to purified human PPARδ LBD. Total binding (○), nonspecific binding (□), and specific binding (●) are shown. The inset shows a Scatchard transformation of the specific binding data. **(b)** Competition binding of GW 2433 to purified human PPARα (□), PPARγ (△), and PPARδ (●) LBDs. Data are the mean of replicate determinations and error bars indicate the range of observations.

respond to a more limited set of natural ligands. A similar conclusion was recently drawn through comparison of the fatty acids that enhance the binding of either PPARα or PPARδ to DNA, presumably through interaction with their LBDs [9]. Therefore, we speculate that although PPARδ is the most widely expressed of the three subtypes, its more limited ligand specificity may result in a specialized physiological function.

Our studies have resulted in the identification of the first high-affinity PPARδ ligand, GW 2433. We have now generated specific radioligands for all three PPAR subtypes: [^{3}H]GW 2331, a PPARα radioligand [8]; [^{3}H]BRL 49653, a PPARγ radioligand [19]; and now [^{3}H]GW 2433, a PPARδ radioligand. These radioligands can be used in competition-binding assays to rapidly screen for new PPAR ligands with improved subtype selectivities. In addition, we have shown that fibrate libraries generated by SPS deliver information about the relative ligand specificity of the PPARs, and may provide opportunities to develop potent and selective ligands for all three subtypes.

Significance

Historically, small-molecule drugs have been found that act on a limited number of protein classes. The nuclear receptor superfamily represents one of these target classes, incorporating the receptors for all of the steroid and retinoid drugs [27]. The existence of a large family of orphan nuclear receptors presents an exciting opportunity for the discovery of new drug molecules that act on these targets [1,28]. Notably, identification of small-molecule ligands is the rate-limiting step in the study of the pharmacology of orphan receptors and in establishing their role in normal physiology and disease processes.

We have presented an approach to the identification of ligands for the orphan nuclear receptors based on the concept of biased chemical libraries. Our work has led to the identification of the first high-affinity PPARδ ligand, GW 2433. The corresponding radioligand [^{3}H]GW 2433 will be an important tool for the identification and characterization of PPAR subtype-selective ligands through competition-binding assays. Finally, our results show that the synthesis of biased chemical libraries containing a fibrate monomer provides a rich source of information about the ligand preferences among the three PPAR subtypes.

Materials and methods

Monomer selection

The total number of commercially available carboxylic acids and isocyanates was reduced by the successive filters of molecular weight >300, incompatible reactive functionality, and multiply functionalized molecules. The resulting 660 carboxylic acids and 154 isocyanates were incorporated into the experimental design. For the design protocol, M1 and either M2 or M3 were held constant as the variable monomer position was evaluated. When held constant the monomer choices for M1, M2 and M3 were fibrate (**2**), phenylacetic acid and phenylisocyante, respectively. A virtual library was generated electronically by splicing together SMILES strings [29] for the fixed monomers and the variable monomer and performing the necessary atom deletions and hybridization changes. A single conformer of each product was generated using CONCORD [30]. After 3D generation of the virtual library of products, each molecule was subjected to assignment of atom types and atomic charges followed by energy minimization using the MVP program [31]. Subsequently, the molecular surface was calculated for each molecule and the electrostatic potential was mapped onto this surface. Five properties were calculated for each molecule: the maximum, minimum, and mean electrostatic potentials, along with the hydrophilic and hydrophobic molecular surface areas. These five properties were standardized and subjected to Spacefill experimental design [18] using the SAS module OPTEX [32] to choose 20 carboxylic acids and eight isocyanates.

Synthesis of the fibrate library

The general method for the synthesis of the fibrate library has been described previously [17]. Sasrin resin (0.89 mmol/g) was loaded with the FMOC amino acids (**1**, **2**, or **3**), and the resulting resins combined in equimolar quantities following FMOC analysis of their relative loading. 20 aliquots (125 mg, 54 μmol each) of the pooled resin (**4**, 0.43 mmol/g) were deprotected with piperidine and then each coupled with one of the carboxylic acids corresponding to M2 (**a–t**). Following reduction with borane–THF complex each resin was subdivided into eight aliquots (15 mg each) and reacted with one of the isocyanates corresponding to M3 (**A–H**). The resulting 160 resins were cleaved with

10% TFA in CH_2Cl_2. The products (**5**) were placed into two microtiter plates in parallel arrays as depicted (Figure 4a) and dried to a thin film. HPLC-MS analysis (Waters NovaPak C18, 3.9 x 100 mm, 10–100% MeCN/0.1% TFA-H_2O, 0.4 ml/min, 220 nM, PE/Sciex API III, positive ion turbospray ionization) indicated that each well generated molecular ions consistent with the predicted products. Dimethylsulfoxide (DMSO) was added to each plate to give a stock solution of 10^{-2} M. The plates were replicated and frozen (–20°C) for storage until biological testing.

Deconvolution of active pools

The FMOC amino acids (**1**, **2**, or **3**) were loaded onto Sasrin resin. SPS was conducted on the individual resins without pooling. The final resins were cleaved and the products purified by solid phase extraction (Bakerbond, SiOH) as described previously [17]. ^{1}H NMR and HPLC analysis (Hypersil ODS C18 3 µm, 4.6 x 60 mm, 0–100% MeCN/0.1% TFA-H_2O) indicated that the final products (**αeB–γnG**, Table 1) were >95% pure.

Radioligand synthesis

A solution of the unsaturated radioligand precursor (**12**; 4.7 mg, 0.8 µmol) in ethyl acetate (1.5 ml) was transferred to a tritiation flask containing 11 mg of 10% Pd-C. The flask contents were stirred with tritium gas (10 Ci) for 1 h. The catalyst was removed by filtration through a Nalgene filter using MeOH. Labile tritium was removed by successive rotary evaporations to dryness with MeOH (3 x 5 ml). The residue (195 mCi) was purified by HPLC (Ultrasphere ODS C18, 250 x 9.4 mm, 60–75% MeCN/0.1% TFA-H_2O, 4 ml/min) to yield 90 mCi of [^{3}H]GW 2433 with a radiochemical purity of 96% and a specific activity of 38 Ci/mmol. [^{3}H]GW 2433 was stored at –20°C as a solution in ethanol at a concentration of 1 mCi/ml.

Characterization of GW 2433

Isolated as a colorless glass. ^{1}H NMR (CD_3OD): δ 7.6 (d, 1H), 7.0–7.5 (m, 7H), 6.8 (d, 2H), 3.6 (t, 2H), 3.2 (t, 2H), 3.1 (t, 2H), 2.55 (t, 2H), 1.9 (m, 2H), 1.55 (s, 6H). Electrospray-MS (*m/z*-H): 579, 581, 583. Analytical HPLC (Hypersil BDS C18 5 µ, 4.2 x 100 mm, 0–100% MeCN/0.1% TFA-H_2O): R_f 7.0 min at 2 ml/min, purity >95% at 254, 220 nm.

PPAR activation assay

The general protocol for the PPAR cell-based reporter gene assay has been described previously [19,33]. The human PPAR-GAL4 chimeric receptor expression constructs were as described previously [8]. The $(UAS)_5$-tk-SPAP reporter plasmid was generated by inserting five copies of a GAL4 DNA-binding element upstream of the minimal promoter of herpes simplex virus thymidine kinase into the pSPAP vector that contains the heat-stable human secreted placental alkaline phosphatase (SPAP) reporter gene [34,35]. CV-1 cells (24 000) were plated into 96-well plates and transfected with 2 ng of receptor expression plasmid, 8 ng of reporter plasmid, 25 ng of β-galactosidase expression plasmid (pCH110, Pharmacia), and 45 ng of carrier plasmid using the Lipofectamine protocol (Life Technologies). After 16 h, the medium was exchanged to DME medium supplemented with 10% delipidated fetal calf serum (Sigma). A 1 µl aliquot of the fibrate library was diluted to a concentration of 5 µM with DME medium and added to the cells. After an additional 24 h, cell extracts were prepared and assayed for alkaline phosphatase activity and β-galactosidase activity using standard colorimetric assays as described previously [19,34]. Alkaline phosphatase activity was corrected for transfection efficiency using the β-galactosidase activity as internal standard. Fold activation relative to vehicle treated control was calculated and expressed as per cent relative to GW 2331 at 10 µM. To generate full concentration–response curves the assay was performed using aliquots of the test compound diluted at 1/2 log concentrations from 10 nM to 10 µM. EC_{50} equals the concentration of compound required to induce 50% of the maximum alkaline phosphatase activity.

Saturation binding of [^{3}H]GW 2433

Using 96-well culture plates, 50 nM purified human PPARδ LBD and the desired concentration of [^{3}H]GW 2433 were diluted to a total volume of 100 µl with buffer consisting of 50 mM KCl, 5 mM EDTA, 10 mM dithiothreitol (DTT), 50 mM HEPES, at pH 7. Saturation binding assays were conducted using concentrations of [^{3}H]GW 2433 ranging from 1 to 250 nM. Nonspecific binding at each concentration of [^{3}H]GW 2433 was estimated in parallel incubations containing 50 µM unlabeled GW 2433. Plates were incubated for 2 h at room temperature. Free ligand was separated from receptor-bound ligand by size exclusion chromatography using commercially available 96-well format spin columns. Samples (50 µl) from each well of a single test plate were loaded onto a buffer- and temperature-equilibrated 96-well gel filtration block (Advanced Genetics Technology Corporation). The block was placed on top of a clean microtiter plate and centrifuged at 1100 g for 4 min. Scintillation fluid (180 µl) was added to each well of the plate containing the eluent, and the plates were sealed and allowed to equilibrate for at least 4 h before counting in a Wallac 1450 Microbeta counter. The amount of nonspecific binding at each concentration of [^{3}H]GW 2433 was subtracted from all wells and plots of [^{3}H]GW 2433 concentration versus counts per minute (cpm) bound were constructed. The K_d values for [^{3}H]GW 2433 were determined from a nonlinear least squares fit of the data to a simple binding model.

Competition-binding assay

Competition-binding curves for GW 2433 were determined with expressed human PPARα, PPARγ and PPARδ LBDs under the same conditions used for the saturation binding experiments. Assays were conducted in the presence of 0.17 nM to 10 µM of GW 2433 in the presence of 20 nM of the specific radioligand. [^{3}H]GW 2331 was employed for PPARα [8], [^{3}H]BRL 49653 for PPARγ [19], and [^{3}H]GW 2433 for PPARδ. The amount of nonspecific binding, as assessed by control wells containing 50 µM of the corresponding unlabeled ligand, was subtracted from each data point. Plots of GW 2433 concentration versus cpm of radioligand bound were constructed. Apparent K_i values were estimated from a nonlinear least squares fit of the data assuming simple competitive binding.

Supplementary material

Details of the synthesis and analytical data for the M1 monomers (**1–3**) and the radioligand precursor (**12**) are published with this paper on the internet.

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