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## Perspective

### The PPARs: From Orphan Receptors to Drug Discovery<sup>†</sup>

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#### Introduction

Dietary fat intake is an environmental factor that affects many aspects of human health.<sup>1</sup> Several lines of evidence suggest that common diseases of modern society are associated with high-fat Western diets combined with a sedentary lifestyle.<sup>2</sup> For example, diabetes, obesity, and cardiovascular disease are major causes of mortality and morbidity whose incidence tracks with the rate of industrialization in many countries.<sup>3</sup> Conversely, it has been established that caloric restriction with low-fat diets in rodents and primates leads to increased longevity and lower incidence of metabolic and cardiovascular disorders.<sup>4</sup> Interestingly not all dietary fat is bad; diets high in monounsaturated fatty acids (the Mediterranean diet) or polyunsaturated fatty acids (the Eskimo diet) appear to have cardioprotective effects.<sup>5,6</sup> The epidemiology of human metabolic diseases and animal feeding studies support the proposal that caloric intake plays an important role in the regulation of lipid metabolism, insulin sensitivity, glucose homeostasis, and atherosclerosis.<sup>1,7</sup> It is therefore not surprising that mammals have evolved with hormonal systems to regulate the physiological response to dietary intake of fatty acids.<sup>8</sup> A family of transcription factors known as the **P**eroxisome **P**roliferator-**A**ctivated **R**eceptors (PPARs) plays a central role in regulating the storage and catabolism of dietary fats. The PPARs were cloned less than a decade ago as orphan members of the nuclear receptor gene family that includes the receptors for the steroid, retinoid, and thyroid hormones.<sup>9,10</sup> This review covers

the rapid progress in the functional analysis of these orphan receptors, research which has led to a greater understanding of the importance of fatty acids as hormones and has established the PPARs as molecular targets for the development of drugs to treat human metabolic diseases.

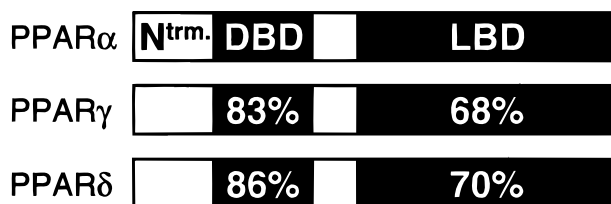
#### Functional Domains and Bioassays

There are three PPAR subtypes, which are the products of distinct genes and are commonly designated PPAR $\alpha$  [NR1C1], PPAR $\gamma$  [NR1C3], and PPAR $\delta$  [NR1C2].<sup>11</sup> The PPARs possess a domain structure common to other members of the nuclear receptor gene family (Figure 1A). Sequence comparison of their DNA-binding domains (DBD) shows that they are highly conserved, while the ligand-binding domains (LBD) have a slightly lower level of conservation across the subtypes. Within the LBD certain conserved amino acids have been mapped to critical receptor functions involved in signal transduction. However, there is significant sequence variation in the residues that line the ligand-binding pocket,<sup>12–14</sup> which is reflected in the fact that each receptor subtype is pharmacologically distinct.<sup>15</sup> The N-terminal region of the receptor shows low sequence identity across the subtypes and is in general less well-characterized. Recent data suggests that the N-terminal domain is responsible for differences in the biological function of the subtypes.<sup>16</sup> In addition, phosphorylation of the PPAR N-terminal domain by mitogen-activated protein kinase can affect the transcriptional activity<sup>17–21</sup> and possibly ligand binding of the receptor.<sup>22</sup> The PPARs form heterodimers with another nuclear receptor, the 9-*cis*-retinoic acid receptor (RXR) [NR2B].<sup>11</sup> The PPAR/RXR heterodimers bind to DNA sequences containing direct repeats of the

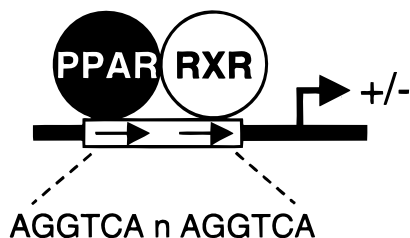
<sup>†</sup> Dedicated to the memory of Gordon L. Hodgson, 1945–2000.

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## A



## B



**Figure 1.** A. Functional domains of the PPARs: N<sup>trm</sup>, N-terminus; DBD, DNA-binding domain; LBD, ligand-binding domain. Numbers represent the percent (%) identity between the human subtypes. B. PPAR/RXR heterodimer binds to a DR-1 response element in the regulatory regions of target genes. When an agonist ligand binds to the heterodimer, recruitment of coactivator proteins (not shown) leads to transcriptional activation.

hexanucleotide sequence AGGTCA separated by one nucleotide, known as DR-1 response elements (Figure 1B).<sup>23</sup> This sequence or minor variants have been characterized within the promoter regions of PPAR target genes such as acyl-CoA oxidase (*AOX*) and the adipocyte fatty acid-binding protein (*aP2*).<sup>24</sup>

In common with other members of the nuclear receptor gene family, the PPARs are ligand-activated transcription factors. The binding of agonist ligands to the receptor results in changes in the expression level of mRNAs encoded by PPAR target genes. This process is known as 'transactivation', and cell-based assays have been developed which monitor this functional activity. Transactivation assays use cells that have been transfected with a vector expressing the receptor as well as a second vector containing a DR-1 response element and a reporter gene, which encodes an enzyme such as chloramphenicol acetyltransferase, secreted placental alkaline phosphatase (SPAP), or firefly luciferase.<sup>25</sup> Activation of the receptor by agonist ligands leads to induction of reporter enzyme expression, which can be conveniently assayed using standard colorimetric or photometric plate readers. Either PPAR or RXR ligands can induce reporter expression, indicating that the PPAR/RXR heterodimer is the functional transcription factor within cells.<sup>23</sup> Although the standard transactivation assay is a powerful and widely used assay, the results can be complicated by the presence of other nuclear receptors within the host cells that bind to the same DR-1 response element as the PPAR/RXR heterodimer. Modified variants of the transactivation assay exploit the observation that the LBD and DBD are autonomous domains, such that they maintain their ligand- or DNA-binding properties when expressed as chimeric proteins with other nuclear receptors or transcription factors.<sup>26</sup> Of particular note is the PPAR-GAL4 transactivation assay, which uses chimeric receptors

**Table 1.** Activity of PPAR Agonists in Cell-Based Transactivation Assays<sup>a</sup>

compound	murine receptor EC <sub>50</sub> (μM)			human receptor EC <sub>50</sub> (μM)		
	PPARα	PPARγ	PPARδ	PPARα	PPARγ	PPARδ
Wy-14643	0.63	32	ia at 100	5.0	60	35
clofibrate <sup>b</sup>	50	~500	ia at 100	55	~500	ia at 100
fenofibrate <sup>b</sup>	18	250	ia at 100	30	300	ia at 100
bezafibrate	90	55	110	50	60	20
GW 9578	0.005	1.5	2.6	0.05	1.0	1.4
troglitazone	ia	0.78	ia	ia	0.55	ia
pioglitazone	ia	0.55	ia	ia	0.58	ia
rosiglitazone	ia	0.076	ia	ia	0.043	ia
KRP-297	10	0.14	7.2	0.85	0.083	9.1
JTT-501 <sup>b</sup>	4.3	0.089	ia	1.9	0.083	ia
SB 213068	0.93	0.10	ia	0.74	0.066	ia
GI 262570	ia	0.00035	ia	0.45	0.00034	ia
GW 1929	ia	0.013	ia	ia	0.0062	ia
GW 7845	ia	0.0012	ia	3.5	0.00071	ia
GW 0207	ia	0.14	ia	ia	0.044	ia
L-796449	7.6	0.010	0.023	0.0041	0.0052	0.0079
L-165041	ia	10	3.8	10	5.5	0.53
GW 2433	0.27	1.5	0.41	0.17	2.5	0.19

<sup>a</sup> All data was generated using the PPAR-GAL4 transactivation assay using an SPAP reporter as described (ref 177); ± 20%, *n* ≥ 3; ia = inactive at 10 μM or the concentration indicated. <sup>b</sup> Data is for the active metabolite.

where the PPAR LBD is fused to the DBD of the yeast transcription factor GAL4 and employs a reporter gene containing a GAL4 response element.<sup>27</sup> Since mammalian cells do not contain GAL4, only the transfected PPAR-GAL4 chimeric receptors can activate the reporter gene, effectively eliminating interference from endogenous nuclear receptors. In general, PPAR agonists show comparable potency and efficacy in assays using either the PPAR-GAL4 chimeras or the full-length receptors. The potency and selectivity of several classes of PPAR agonists in the PPAR-GAL4 assay are presented in Table 1.

Biochemical and genetic studies of the mechanism of transcription regulation have identified a series of accessory proteins that bind to nuclear receptors in a ligand-dependent manner.<sup>28,29</sup> These so-called 'coactivator' proteins promote the initiation of transcription and can be loosely categorized into three classes: proteins with histone acetylase activity that remodel chromatin structure (e.g. SRC1<sup>30,31</sup> and CBP/p300<sup>32</sup>), members of the DRIP/TRAP complex that interact with the basal transcriptional machinery (e.g. PBP/TRAP220<sup>33,34</sup>), and those proteins with poorly defined function (e.g. PGC1,<sup>35</sup> RIP140,<sup>36,37</sup> and ARA70<sup>38</sup>). Cell-free ligand sensing assays have been developed that exploit the ligand-dependent recruitment of coactivator proteins to the receptor. The coactivator-dependent receptor ligand assay (CARLA) detects the binding of a <sup>35</sup>S-labeled fragment of SRC1 to the PPAR LBD on an acrylamide gel.<sup>39</sup> Recently, the use of fluorescence resonance energy transfer has allowed the development of homogeneous CARLA assays.<sup>40</sup> These assays can be used as high-throughput cell-free screens for the identification of new PPAR ligands and for the characterization of coactivator recruitment to the receptor.

Several radioligands are now available for use in conventional competition binding assays: [<sup>3</sup>H]GW 2331 for PPARα,<sup>41</sup> [<sup>3</sup>H]rosiglitazone (BRL49653),<sup>27</sup> [<sup>3</sup>H]AD-5075,<sup>42</sup> and [<sup>125</sup>I]SB-236636<sup>43</sup> for PPARγ; and [<sup>3</sup>H]GW 2433<sup>44</sup> and [<sup>3</sup>H]L-783483<sup>45</sup> for PPARδ. Each of these radioligands is reported to show specific binding to the

corresponding PPAR subtype. Initial PPAR binding assays used gel filtration to separate the bound radioligand from the free ligand.<sup>27,41</sup> However this technique is not ideal for high-throughput screening. The use of scintillation proximity assay (SPA) technology has greatly increased the throughput of PPAR competition binding assays.<sup>46,47</sup> SPA beads emit light when held in close proximity to a suitable radionuclide (e.g. <sup>3</sup>H or <sup>125</sup>I). If the receptor is attached to the SPA bead, binding of a radioligand to the PPAR LBD leads to a readily detectable signal. Displacement of the radioligand by a test compound leaves the free radioligand in solution, where it can no longer promote emission by the SPA bead. This technology removes the need to separate free radioligand from the bound ligand, greatly simplifying automated high-throughput screening. The homogeneous format of the SPA binding assay allows determination of equilibrium binding affinities and also permits the use of relatively low-affinity radioligands.<sup>46</sup>

The biology of the PPARs has been driven, in large part, by the availability of potent and selective ligands for the receptors.<sup>48</sup> Through the use of binding and functional assays, several groups have reported the identification and optimization of PPAR ligands for each of the three subtypes (Table 1). These chemical tools have been used in a "reverse endocrinology" approach to uncover the role of the PPARs in human physiology and disease processes.<sup>49</sup>

### PPAR $\alpha$

PPAR $\alpha$  was originally cloned from a mouse liver cDNA library<sup>9</sup> and has since been cloned from frogs,<sup>50</sup> rats,<sup>26</sup> guinea pigs,<sup>51</sup> and humans.<sup>52,53</sup> Although the DBDs are identical across a variety of species, the LBDs exhibit lower homology, which may reflect evolutionary adaptation to different dietary ligands. Comparison of human PPAR $\alpha$  to the murine PPAR $\alpha$  shows an 85% identity at the nucleotide level and 91% identity at the amino acid level. The human PPAR $\alpha$  gene has been mapped to chromosome 22q12-q13.1 by somatic cell hybridization and linkage analysis.<sup>52</sup> Analysis of PPAR $\alpha$  tissue distribution in rodents and humans revealed high levels of expression in metabolically active tissues, such as liver, heart, kidney, and muscle.<sup>54,55</sup> PPAR $\alpha$  mRNA levels are under diurnal control of endogenous glucocorticoids in at least some of these tissues.<sup>56,57</sup>

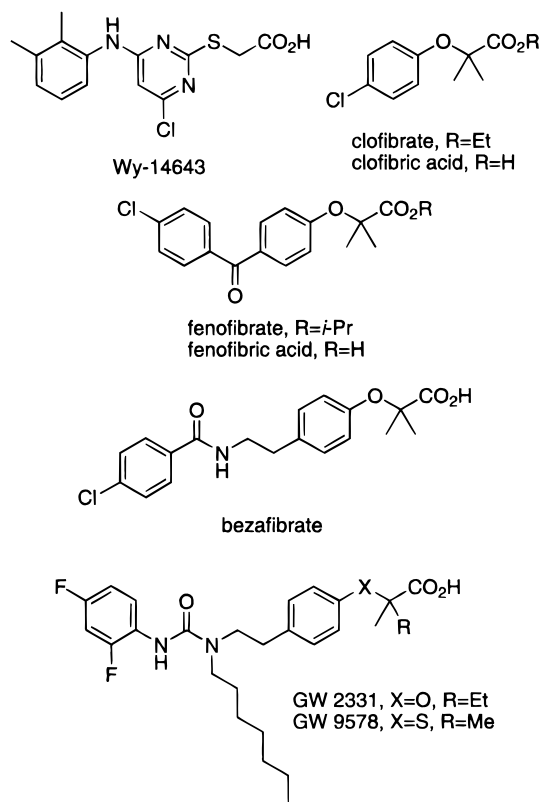
PPAR $\alpha$  is activated by a diverse range of compounds, such as fibrates and plasticizers, which cause the proliferation of peroxisomes and hepatomegaly in rodents.<sup>9</sup> This phenomenon has not been observed in nonrodent species, including humans, even though compounds that cause peroxisome proliferation activate both human and murine PPAR $\alpha$  in cell-based transactivation assays.<sup>58</sup> Thus, although administration of peroxisome proliferators to rodents results in hepatomegaly and hepatocellular proliferation accompanied by suppression of apoptosis,<sup>59</sup> years of clinical experience with fibrates in humans has not led to evidence of peroxisome proliferation, as determined by morphological examination of liver biopsies,<sup>60,61</sup> or to an increased incidence of liver cancer.<sup>62–64</sup> The molecular basis of this species-specific response may be due to differences in the function of PPAR $\alpha$  in rodents and humans. For example, there are differences in the hepatic expression

of PPAR $\alpha$  across species. The wild-type PPAR $\alpha$  is expressed in rodent livers at 10 times higher levels than in human livers.<sup>65</sup> In addition, a splice-variant of human PPAR $\alpha$ , which represents 20–50% of the total PPAR $\alpha$  mRNA in the liver, has been identified in which exon 6 is deleted.<sup>65,66</sup> The truncated human receptor lacks a LBD and was also unable to bind to a DR-1 response element. Although the truncated isoform inhibited the activity of wild-type receptor under certain conditions, immunolocalization showed that it was not normally present in the nucleus of cells.<sup>66</sup> A homologous truncated PPAR $\alpha$  transcript could not be detected in rodent liver. Differences in the expression levels of PPAR $\alpha$  and its isoforms may be only one factor affecting the species-specific response to peroxisome proliferators. It has been noted that the DR-1 response elements of key peroxisomal genes such as *AOX* are not conserved between rodents and humans.<sup>67</sup> Therefore, the physiological role of PPAR $\alpha$  as a regulator of peroxisome function appears to be restricted to rodents, and the common designation of this receptor (and the other PPAR subtypes) does not reflect its biological function in humans.

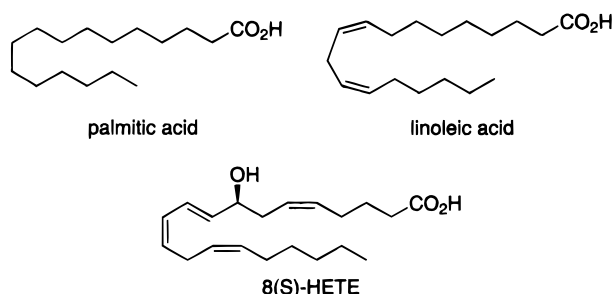
**Synthetic Ligands.** The hypolipidemic fibrate drugs are an important class of PPAR $\alpha$  ligands. Wy-14643 was identified as a micromolar activator of murine PPAR $\alpha$  using a cell-based transactivation assay.<sup>9</sup> This drug as well as clofibrate, fenofibrate, and bezafibrate were developed as hypolipidemic agents through optimization of their lipid-lowering activity in rodents before the discovery of the PPARs. Table 1 shows the potencies of several fibrate drugs on the human and murine PPARs. Clofibric acid and fenofibric acid, the active metabolites of clofibrate and fenofibrate, are dual activators of PPAR $\alpha$  and PPAR $\gamma$ , with ~10-fold selectivity for PPAR $\alpha$ , while bezafibrate activates all three PPAR subtypes at comparable doses. All of these compounds require high micromolar concentrations to activate human PPAR $\alpha$ , which may explain why high doses (300–1200 mg/day) are required for their clinical activity.<sup>68</sup> Attempts to identify more potent compounds led to the synthesis of a series of ureidofibrates that were active at lower doses in rodent models of hyperlipidemia.<sup>69</sup> One of these ureidofibrates, GW 2331, was shown to bind to both PPAR $\alpha$  and PPAR $\gamma$  with nanomolar affinity.<sup>41</sup> Chemical libraries of ureidofibrates have been synthesized on solid-phase,<sup>70</sup> which has enabled this class of ligands to be rapidly optimized for PPAR activity on the human receptors.<sup>44</sup> The closely related ureidothioisobutyric acid GW 9578 has recently been reported as a potent and subtype-selective PPAR $\alpha$  agonist with improved lipid-lowering activity compared to fenofibrate.<sup>71</sup>

**Natural Ligands.** Several groups have implicated fatty acids as natural ligands for PPAR $\alpha$ . The Gustafsson laboratory was the first to demonstrate that a range of saturated and unsaturated fatty acids could activate PPAR $\alpha$ .<sup>26</sup> A search for natural PPAR $\alpha$  ligands in human serum identified palmitic acid, oleic acid, linoleic acid, and arachidonic acid as endogenous activators of rat PPAR $\alpha$ .<sup>72</sup> Direct binding of fatty acids to PPAR $\alpha$  has been demonstrated using competition binding assays<sup>14,41</sup> and cell-free ligand sensing assays.<sup>39,73</sup> Notably, PPAR $\alpha$  is the only subtype that binds to a wide range of saturated fatty acids.<sup>14</sup> All of the fatty acids identified to date bind to PPAR $\alpha$  with affinities in the micromolar





range. While total fatty acid levels in serum reach these concentrations,<sup>74</sup> it is not known whether the free concentrations of fatty acids in cells are high enough to activate the receptor. This has prompted several investigators to search for high-affinity natural ligands among the known eicosanoid metabolites of polyunsaturated fatty acids. The lipoxygenase metabolite 8(*S*)-HETE was identified as a higher-affinity PPAR $\alpha$  ligand,<sup>39,41,73,75</sup> although it is not found at sufficiently high concentrations in the correct tissues to be characterized as a natural ligand. Since no single high-affinity natural ligand has been identified, we and others have proposed that one physiological role of PPAR $\alpha$  may be to sense the total flux of fatty acids in metabolically active tissues.<sup>14,26,39,41,73</sup>



**PPAR $\alpha$  and Dyslipidemia.** The discovery that the fibrates activate PPAR $\alpha$  has triggered extensive research into the role of this receptor in mediating the lipid-lowering activity of these drugs. In humans, fibrates are effective at lowering serum triglycerides and raising HDL cholesterol (HDLc), primarily through increased clearance and decreased synthesis of triglyceride-rich VLDL.<sup>76</sup> Fibrates lower serum levels of apoC-III, a known inhibitor of VLDL clearance,<sup>77</sup> in both

humans<sup>78</sup> and rodents.<sup>79</sup> A PPAR response element has been identified in the proximal promoter of the *apoC-III* gene through which PPAR $\alpha$  can inhibit its expression.<sup>80</sup> Response elements have also been identified in proximal promoters of other genes involved in lipid metabolism, such as *AOX*,<sup>50</sup> cytochrome P450 4A,<sup>81</sup> liver-fatty acid-binding protein (*L-FABP*),<sup>82</sup> and lipoprotein lipase (*LPL*).<sup>83</sup> In rodents apoA-I expression is down-regulated by fibrate treatment, leading to a reduction in HDLc. Since rodents carry most of their cholesterol in the HDL fraction, this is one pathway by which fibrates lower cholesterol levels in rodents. In humans, however, the reverse is true: fibrate treatment increases apoA-I expression leading to a rise in HDLc levels. This difference in gene regulation may be due to variations in the response element sequences in the rodent and human *apoA-I* genes.<sup>84–86</sup> The availability of a PPAR $\alpha$  null mouse has aided the study of the role of the receptor in lipid homeostasis.<sup>87</sup> In contrast to wild-type mice, PPAR $\alpha$ <sup>−/−</sup> mice do not show decreases in serum lipid levels as a consequence of treatment with fibrates. Moreover, the null mice do not exhibit hepatomegaly, peroxisome proliferation, or induction of enzymes responsible for fatty acid oxidation when challenged with fibrates.<sup>88</sup>

The LDL cholesterol (LDLc)-lowering activity of the current fibrate drugs is weak compared to the statin class of drugs that inhibit HMG-CoA reductase.<sup>89</sup> At present, LDLc lowering is the primary endpoint by which lipid-lowering therapies are compared. Data are available showing a correlation of cardiac events with increasing LDLc levels, and cardiovascular outcome studies with statins have shown improvements in patient mortality and morbidity.<sup>90</sup> However, recent evidence has suggested that hypertriglyceridemia and low HDLc levels should also be considered when assessing a patient's cardiovascular risk factors.<sup>91</sup> Fibrates have been shown to slow the progression of atherosclerosis and reduce the number of coronary events in secondary prevention studies<sup>92–94</sup> and in patients with normal levels of LDLc.<sup>95</sup> Potent subtype-selective PPAR $\alpha$  agonists, such as GW 9578, are more effective than the current fibrate drugs at lowering apoC-III levels in rodents.<sup>71</sup> Thus, drugs with potent activity on human PPAR $\alpha$  may be useful adjuncts to current therapies for treatment of dyslipidemias in patients at risk of cardiovascular disease.

**PPAR $\alpha$  and Atherosclerosis.** Factors other than dyslipidemia contribute to the development of coronary heart disease. Evidence is emerging that PPAR $\alpha$  agonists may have direct effects in the arterial wall, which could contribute to the beneficial effects of these drugs in atherosclerosis prevention studies.<sup>92–95</sup> Atherosclerotic lesion formation requires recruitment of monocytes into the arterial wall through expression of adhesion molecules by activated endothelial cells.<sup>96</sup> Expression of the adhesion molecule VCAM-1 was down-regulated by PPAR $\alpha$  agonists in human vascular endothelial cells.<sup>97</sup> This process was mediated in part by inhibition of NF- $\kappa$ B, a ubiquitous transcription factor that transduces the effects of many proatherogenic and inflammatory stimuli.<sup>98</sup> PPAR $\alpha$  has also been shown to inhibit the actions of NF- $\kappa$ B in aged mice. Wy-14643 corrected the abnormal expression of genes under the control of

NF- $\kappa$ B in these mice<sup>99</sup> but was ineffective in aged *PPAR $\alpha$ <sup>-/-</sup>* mice.<sup>100</sup> Inflammatory processes have been implicated in disruption of the atherosclerotic plaque that leads to thrombotic events.<sup>101</sup> PPAR $\alpha$  agonists were shown to inhibit the IL-1-stimulated release of IL-6 and inflammatory prostaglandins in vascular smooth muscle cells.<sup>102</sup> Patients with coronary artery disease also responded favorably to fenofibrate treatment, showing reduced plasma levels of IL-6, fibrinogen, and C-reactive protein,<sup>102</sup> possibly through negative regulation of NF- $\kappa$ B and AP-1 by PPAR $\alpha$ .<sup>103</sup> Additional evidence that PPAR $\alpha$  can affect inflammatory processes was generated using the classical mouse ear-swelling test. Arachidonic acid-induced ear swelling lasted longer in *PPAR $\alpha$ <sup>-/-</sup>* mice compared to wild-type mice,<sup>104</sup> and it was hypothesized that PPAR $\alpha$  may regulate genes involved in the catabolism of inflammatory eicosanoids. In support of this proposal the ear-swelling response to phorbol myristate acetate, which is not mediated by eicosanoids, was not affected by PPAR $\alpha$  genotype.<sup>104</sup>

**PPAR $\alpha$  and Obesity/Diabetes.** Obesity is a major risk factor for the development of diabetes, and fibrate treatment has been reported to reduce weight gain in rodents without effects on food intake.<sup>105–108</sup> Notably, *PPAR $\alpha$ <sup>-/-</sup>* mice show increased accumulation of body fat as they age.<sup>109,110</sup> These observations suggest that PPAR $\alpha$  may affect body weight through regulation of fatty acid catabolism or energy expenditure.<sup>111</sup> Uncoupling proteins (UCP) 1–3 are mitochondrial membrane transporters that uncouple substrate oxidation from ATP synthesis, allowing conversion of fuel into heat.<sup>112</sup> Examination of the changes in UCP mRNA expression following bezafibrate treatment of rodents revealed increased levels of UCP1 in white adipose tissue (WAT) and increased levels of UCP3 in WAT and skeletal muscle.<sup>113</sup> In rat neonates Wy-14643 has been shown to induce the expression of *UCP3*,<sup>114</sup> a gene whose message levels are reduced in type 2 diabetics.<sup>115</sup> Three putative PPAR response elements have been identified in the *UCP3* promoter sequence.<sup>116</sup> Wy-14643 has been shown to transactivate the *UCP1* promoter in fetal rat brown adipocytes, although this did not result in increased UCP1 mRNA levels.<sup>117</sup> Under these same conditions, the PPAR $\gamma$  agonist rosiglitazone activated the *UCP1* promoter and increased UCP1 mRNA levels, but a combination of the PPAR $\alpha$  and PPAR $\gamma$  agonists did not lead to increased UCP1 expression.<sup>117</sup> Interestingly, clofibrate and bezafibrate have been shown to improve glucose tolerance in type 2 diabetic patients.<sup>118–120</sup> However, the clinically used fibrates are only moderately selective for PPAR $\alpha$  over PPAR $\gamma$ ; thus it is not clear whether activation of the latter subtype is responsible for the observed effects. Confirmation of the therapeutic potential of PPAR $\alpha$  agonists in obesity or diabetes will require clinical testing of drugs with greater subtype selectivity than the currently available fibrates.

## PPAR $\gamma$

PPAR $\gamma$  is the most extensively studied of the three PPAR subtypes to date. The receptor has been cloned from a number of species, including salmon,<sup>121</sup> mice,<sup>15,122</sup> hamsters,<sup>123</sup> frogs,<sup>50</sup> pigs,<sup>124</sup> rhesus monkeys,<sup>125</sup> and humans.<sup>126–128</sup> The human PPAR $\gamma$  protein is homolo-

gous to the murine PPAR $\gamma$  protein, with 95% identity at the amino acid level. In fact, the PPAR $\gamma$  protein shows a remarkable conservation across all the species from which it has been cloned, in contrast to that found thus far for PPAR $\alpha$  and PPAR $\delta$ . This high level of conservation may reflect the pivotal role that PPAR $\gamma$  plays as a regulator of glucose and lipid homeostasis, essential functions across all species. The human *PPAR $\gamma$*  gene has nine exons that extend over more than 100 kb of genomic DNA and has been mapped to human chromosome 3p25 by somatic cell hybridization and linkage analysis.<sup>126,129</sup>

Three mRNA isoforms of PPAR $\gamma$  have been detected in humans (termed PPAR $\gamma$ 1, PPAR $\gamma$ 2, and PPAR $\gamma$ 3) which arise as products of different promoter usage.<sup>130–132</sup> PPAR $\gamma$ 1 and PPAR $\gamma$ 3 mRNAs code for the same protein, while PPAR $\gamma$ 2 codes for a different protein containing 28 additional amino acids at the N-terminus. Analysis of the tissue distribution of the three human PPAR $\gamma$  isoforms revealed some differences. PPAR $\gamma$ 1 had the broadest tissue expression, while the PPAR $\gamma$ 2 and PPAR $\gamma$ 3 isoforms had a more restricted distribution. Thus, PPAR $\gamma$ 2 was expressed predominantly in adipose tissue,<sup>130</sup> and PPAR $\gamma$ 3 was found only in adipose tissue, macrophages, and colon epithelium.<sup>132,133</sup> In addition to the aforementioned tissues, PPAR $\gamma$ 1 mRNA was found in the heart, large and small intestines, colon, kidney, pancreas, spleen, and skeletal muscle. Cell culture experiments have demonstrated that PPAR $\gamma$ 2 mRNA was induced during the early stages of adipocyte differentiation,<sup>134–136</sup> but PPAR $\gamma$ 1 mRNA remained unchanged.<sup>137</sup> However, the functional significance of the multiple isoforms of PPAR $\gamma$  is currently unclear.<sup>131,136,138</sup>

PPAR $\gamma$  is a critical transcription factor in the regulation of adipocyte differentiation. Forced expression of PPAR $\gamma$  in fibroblasts in the presence of weak PPAR $\gamma$  activators resulted in differentiation of the cells to adipocytes.<sup>135</sup> Adipogenesis appears to require coordinated expression of the PPAR $\gamma$  and two other groups of transcription factors, C/EBP and ADD-1/SREBP-1. The detailed aspects of the interplay between PPAR $\gamma$  and these other transcription factors in adipocyte differentiation have been reviewed recently.<sup>139,140</sup> Adipocyte differentiation is accompanied by the induction of several genes involved in lipid homeostasis such as *aP2*,<sup>136</sup> *PEPCK*,<sup>141</sup> acyl-CoA synthetase (*ACS*),<sup>142</sup> and *LPL*.<sup>83</sup> All of these genes have been shown to contain PPAR response elements in their regulatory regions. PPAR $\gamma$  has also been shown to up-regulate the expression of the fatty acid transporters FATP-1 and CD36 in adipocytes.<sup>143,144</sup> These data demonstrate that PPAR $\gamma$  plays a pivotal role in the adipogenic signaling cascade and also suggest that the receptor can influence the production and cellular uptake of its own activators.

PPAR $\gamma$  has been shown to modulate a number of other genes involved in energy storage and utilization. Activation of PPAR $\gamma$  represses the expression of the *ob* gene, which codes for leptin.<sup>145,146</sup> PPAR $\gamma$  also appears to oppose the effects of another important adipocyte signaling factor, TNF $\alpha$ . Treatment of obese animals with PPAR $\gamma$  agonists reduces adipose tissue expression of TNF $\alpha$ .<sup>147</sup> PPAR $\gamma$  has been shown to up-regulate expression of the mitochondrial uncoupling proteins

UCP1, UCP2, and UCP3 in cells<sup>148–151</sup> and animals.<sup>152,153</sup> Recently, it was shown that activation of PPAR $\gamma$  in cultured adipocytes induced expression of c-Cbl associated protein, a potential signaling protein in insulin action.<sup>154</sup> The growing list of genes that are modulated by PPAR $\gamma$  continues to reinforce the notion that PPAR $\gamma$  is a key regulator of adipocyte function and systemic lipid homeostasis.

**Synthetic Agonists.** The first compounds reported as high-affinity PPAR $\gamma$  agonists were a class of antidiabetic agents known as thiazolidinediones (TZDs) or "glitazones".<sup>27</sup> The TZDs had been developed over a period of 15 years through empirical compound screening in rodent models of insulin resistance.<sup>155</sup> The molecular mechanism of action of the TZDs remained unknown until several reports in the mid-1990s suggested a possible connection between these agents and the PPARs. TZDs had been shown to induce gene expression in adipocytes and to enhance adipocyte differentiation in cell culture.<sup>156,157</sup> The TZD pioglitazone was also shown to increase expression of the *aP2* gene,<sup>158</sup> through a region of its promoter that had been independently identified by the Spiegelman group as a PPAR $\gamma$  binding site.<sup>136</sup> Through the use of binding and transactivation assays, the TZD rosiglitazone (BRL 49653) was identified as a high-affinity subtype-selective agonist for PPAR $\gamma$ .<sup>27,159</sup> In addition, the rank order of PPAR $\gamma$  potency of a number of TZDs has been shown to closely match their glucose-lowering activity in rodents.<sup>42,160</sup> These latter two pieces of data provide compelling evidence that PPAR $\gamma$  is the major receptor mediating the antidiabetic activity of the TZDs.

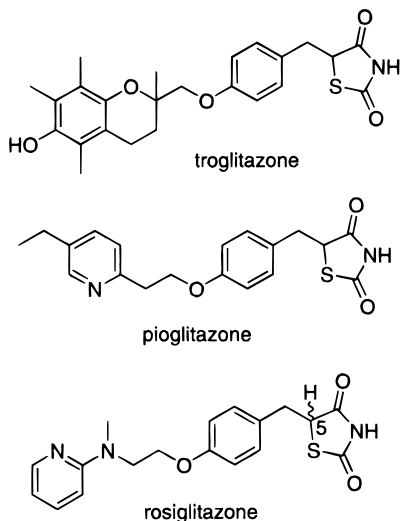
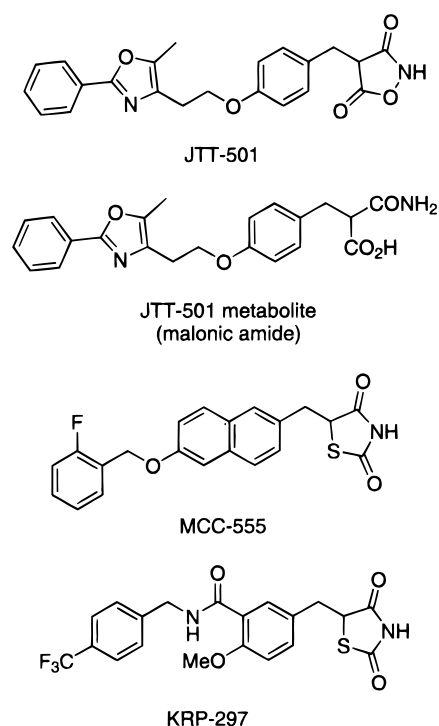


Table 1 lists the potency of those TZDs whose PPAR $\gamma$  activity has been reported. TZDs, in general, are selective for PPAR $\gamma$  over the PPAR $\alpha$  and PPAR $\delta$  subtypes,<sup>27,160</sup> although a TZD, KRP-297, with agonist activity at PPAR $\alpha$  and PPAR $\gamma$  was recently disclosed.<sup>161–163</sup> The structurally related isoxazolidinedione JTT-501 has also been reported to activate PPAR $\alpha$  at concentrations approximately 10-fold higher than those required for activation of PPAR $\gamma$ .<sup>164</sup> Its activity is likely to be mediated through a malonic amide metabolite that is generated by hydrolysis of the heterocyclic ring.<sup>165</sup> The TZD MCC-555 was reported to be a low-affinity but high-efficacy PPAR $\gamma$  agonist that showed potent an-

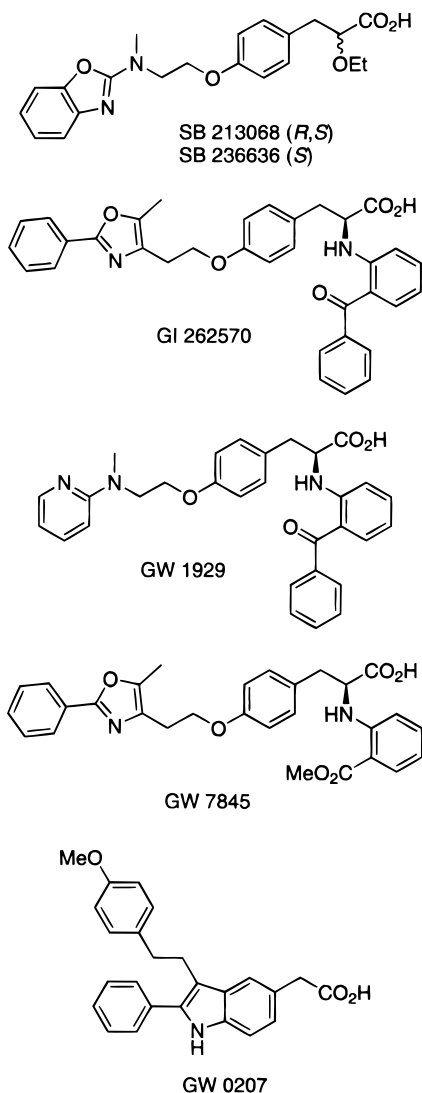
tidiabetic activity in rats.<sup>166,167</sup> Interestingly, MCC-555 appeared to function as a full or partial PPAR $\gamma$  agonist depending on the cell type and the response element used in the transactivation assay.<sup>168</sup> A series of antidiabetic  $\Delta^5$ -unsaturated-TZDs have also been reported, which were claimed to show little or no activity at PPAR $\gamma$ .<sup>169,170</sup> These observations raise the possibility that some TZDs mediate their antidiabetic activity through mechanisms other than PPAR $\gamma$ , although activation of PPAR $\gamma$  by metabolites of these drugs cannot be ruled out. An alternative explanation is that these compounds are able to produce tissue or promoter-specific modulation of PPAR $\gamma$  target genes, which was not detected by the standard PPAR $\gamma$  transactivation assays. Should this latter hypothesis prove valid, it will add a new dimension to the development of PPAR $\gamma$  ligands, similar to that stimulated by the selective estrogen receptor modulators in the osteoporosis field.<sup>171</sup>



The TZDs contain a stereogenic center at C-5 of the heterocyclic headgroup but have been developed as racemates since they undergo racemization under physiological conditions.<sup>172</sup> Using a PPAR $\gamma$  binding assay it has been shown that only the (*S*)-enantiomers of the TZDs bind to the receptor with high affinity.<sup>173</sup> This surprising result suggests that only 50% of the drug substance in the currently approved TZDs binds to the target receptor, while 50% of the drug substance is inactive. To overcome this problem several groups have identified acyclic headgroups that are less prone to racemization. SB 213068 and its (*S*)-isomer, SB 236636, are representatives of a series of  $\alpha$ -alkoxy- $\beta$ -phenylpropanoic acids<sup>174,175</sup> that show agonist activity at PPAR $\gamma$  and PPAR $\alpha$  (Table 1). Within this series the (*S*)-enantiomers were shown to have higher binding affinity for PPAR $\gamma$  and were more potent than their (*R*)-enantiomers in adipocyte differentiation assays.<sup>43,176</sup> Recently we reported a series of tyrosine-based PPAR $\gamma$  agonists exemplified by GI 262570, GW 1929, and GW



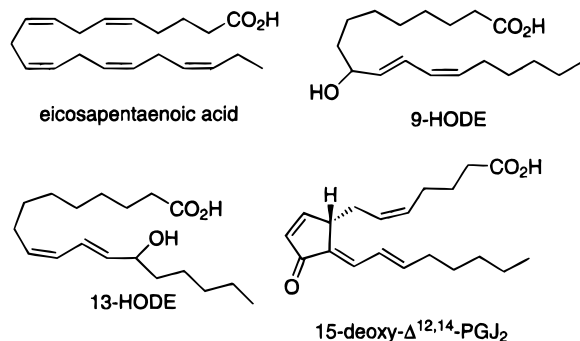
7845.<sup>177–179</sup> These compounds were the first antidiabetic drugs to be optimized based on their activity at human PPAR $\gamma$ . This series also contains some of the most potent PPAR $\gamma$  agonists reported to date, with a number of analogues having sub-nanomolar activity at human PPAR $\gamma$  (Table 1). In addition, these compounds showed >1000-fold selectivity for PPAR $\gamma$  over the PPAR $\alpha$  and PPAR $\delta$  subtypes in cell-based transactivation assays. GW 1929 demonstrated antihyperglycemic activity equal to troglitazone at >100-fold lower plasma concentrations in ZDF rats,<sup>177,180</sup> which parallels their differences in PPAR $\gamma$  binding and activation. Within this series the (*S*)-enantiomers, synthesized from naturally occurring L-tyrosine, were shown to have greater binding affinity and functional activity at PPAR $\gamma$  than the corresponding (*R*)-enantiomers.<sup>177</sup>



Other structurally diverse PPAR $\gamma$  agonists have been described in the literature. The 2,3-disubstituted indole-5-acetic acid derivative GW 0207 was a potent and selective PPAR $\gamma$  agonist.<sup>181</sup> Berger<sup>45</sup> has reported a series of phenylacetic acid derivatives, such as L-796449, which showed potent activity at PPAR $\gamma$ . Notably, L-796449 activated human PPAR $\alpha$  and PPAR $\delta$  at similar concentrations (Table 1), the first reported example of a potent agonist across all three receptor

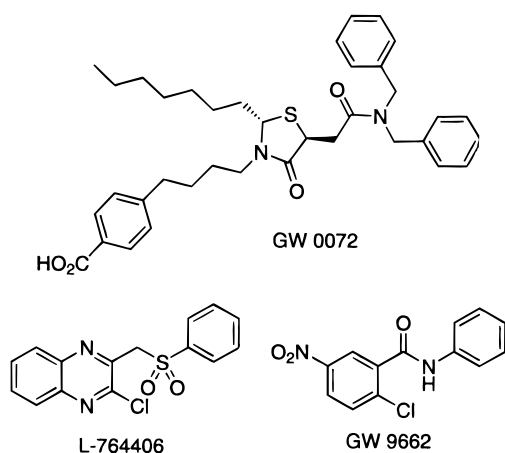
subtypes. Several inhibitors of eicosanoid biosynthesis or receptor signaling pathways have also been reported to be weak agonists at PPAR $\gamma$ . The LTD $_4$  receptor antagonist LY 171883 activated PPAR $\gamma$  in transactivation assays at micromolar concentrations.<sup>15,159</sup> The cyclooxygenase inhibitor indomethacin has been shown to bind and activate PPAR $\gamma$  at high micromolar concentrations.<sup>182</sup> Indomethacin was shown to promote adipocyte differentiation at concentrations similar to that required for activation of PPAR $\gamma$ .<sup>183,184</sup> Several other NSAIDs, including ibuprofen, fenoprofen, and flufenamic acid, were also shown to be weak PPAR $\gamma$  agonists.<sup>182</sup> It is important to note that interaction of these compounds with PPAR $\gamma$  occurs at higher concentrations than is required for inhibition of cyclooxygenase or LTD $_4$  receptor antagonism.

**Natural Ligands.** The search for the natural PPAR $\gamma$  ligands has led to the discovery of a number of fatty acids and eicosanoid derivatives that bind and activate the receptor at micromolar concentrations. Unlike the PPAR $\alpha$  subtype, PPAR $\gamma$  has a clear preference for polyunsaturated acids.<sup>14</sup> The essential fatty acids linoleic acid, linolenic acid, arachidonic acid, and eicosapentaenoic acid (EPA) have been shown to bind PPAR $\gamma$  at micromolar concentrations.<sup>14,41</sup> These values are within the range of concentrations of free fatty acids found in human serum;<sup>74</sup> however, as discussed earlier, it is unclear if these concentrations of fatty acids exist within cells. In fact, fatty acids are not particularly efficacious activators of PPAR $\gamma$ , and it has been demonstrated that intracellular conversion of fatty acids to eicosanoids, through enhanced expression of 15-lipoxygenase, resulted in increased PPAR $\gamma$ -mediated transactivation.<sup>185</sup> The 15-lipoxygenase metabolites of linoleic acid, 9-HODE and 13-HODE, have been shown to function as micromolar PPAR $\gamma$  agonists.<sup>186</sup> Therefore, metabolic conversion of polyunsaturated fatty acids within cells could provide an additional level of hormonal regulation of PPAR $\gamma$ . The J-series of prostaglandins derived from PGD $_2$  have also been identified as PPAR $\gamma$  ligands.<sup>75</sup> The terminal metabolite 15-deoxy- $\Delta^{12,14}$ -prostaglandin J $_2$  (15d-PGJ $_2$ ) activated PPAR $\gamma$  at low micromolar concentrations and also induced adipocyte differentiation.<sup>159,187</sup> This prostaglandin has become the most widely utilized naturally occurring PPAR $\gamma$  ligand. However, it is important to note that 15d-PGJ $_2$  mediates some of its effects in cells through PPAR $\gamma$ -independent signaling pathways,<sup>188–191</sup> and caution must be exercised in attributing the biological effects elicited by 15d-PGJ $_2$  solely to activation of PPAR $\gamma$ .

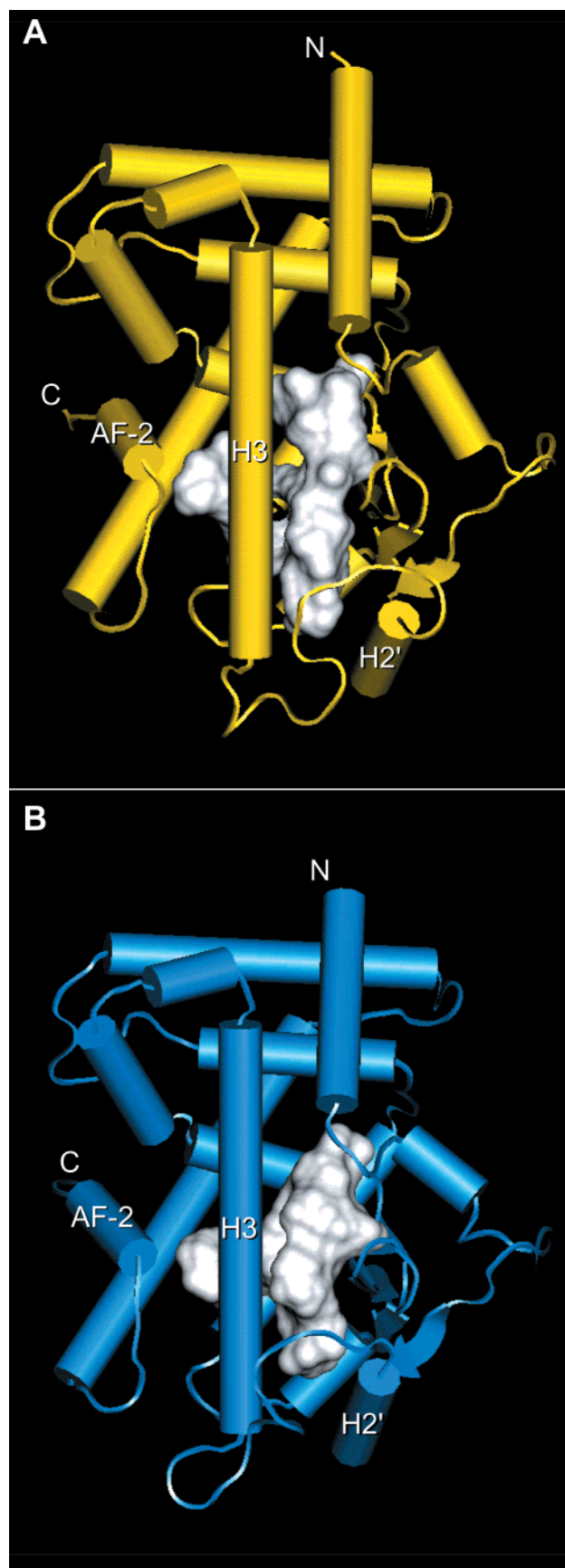


**Partial Agonists and Antagonists.** A novel PPAR $\gamma$  ligand GW 0072, which was identified from a diverse

combinatorial library, profiled as a partial agonist in transactivation assays and was an inhibitor of adipocyte differentiation in cell culture.<sup>192</sup> This compound had only 15–20% of the efficacy of rosiglitazone and was able to antagonize rosiglitazone in transactivation assays to the level of its own partial agonist activity with an  $IC_{50} = 150$  nM. The low efficacy of GW 0072 was paralleled by its reduced ability to recruit coactivator proteins CBP and SRC1 to the receptor, which appears to be the result of a novel binding mode of the ligand within the PPAR $\gamma$  LBD (see below).<sup>192</sup> Although not a pure antagonist, GW 0072 may provide a valuable chemical tool for dissecting the pharmacology of PPAR $\gamma$ . Scientists at Merck have described a partial agonist, L-764406, which is an irreversible PPAR $\gamma$  ligand.<sup>47</sup> L-764406 binds covalently to Cys<sup>285</sup> on helix 3 of the PPAR $\gamma$  LBD (Cys<sup>313</sup> in PPAR $\gamma$ 2). L-764406 displayed approximately 25% of the maximal activity obtained with TZDs, both in transactivation assays and in the induction of aP2 expression in 3T3-L1 preadipocyte cells. Antagonist activity of this compound was not reported. However, we have shown that an irreversible PPAR ligand, GW 9662, was able to antagonize the activation of PPAR $\gamma$  in macrophages.<sup>185</sup>

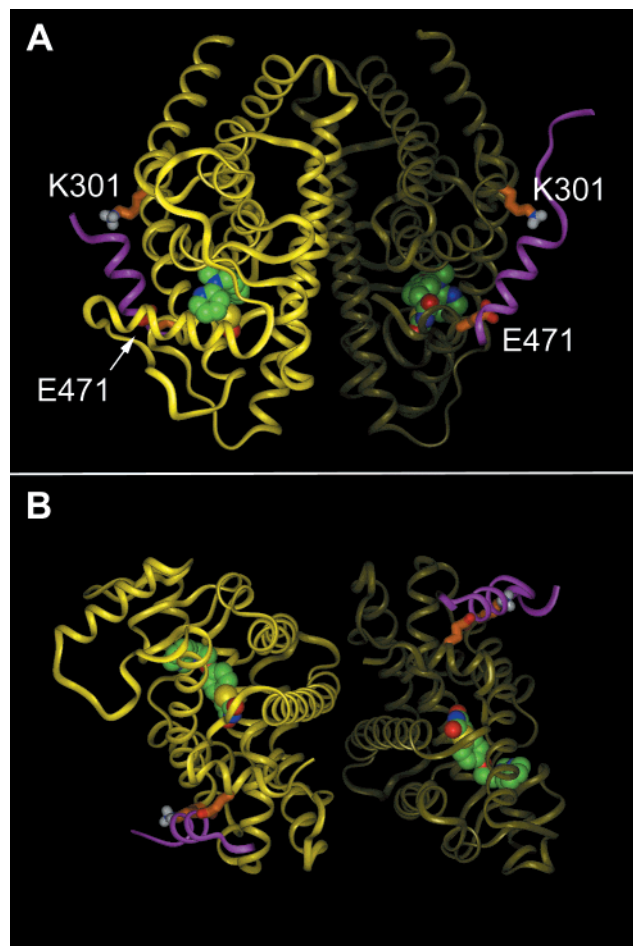


**Protein Structure.** The structure of the PPAR $\gamma$  LBD has been determined by X-ray crystallography. Structures have been solved in the absence of ligand (apo-PPAR $\gamma$ )<sup>12,13</sup> (Figure 2A) and bound to the TZD rosiglitazone together with an 88-amino acid fragment of the coactivator SRC1 (Figure 3).<sup>12</sup> The PPAR $\gamma$  LBD is a bundle of 13  $\alpha$ -helices and a small four-stranded  $\beta$ -sheet (Figure 2A), with an overall fold similar to other nuclear receptor structures from helix 3 to the C-terminus.<sup>193</sup> The crystal structure of the apo-PPAR $\gamma$  LBD revealed a large ( $\sim 1300$  Å<sup>3</sup>) Y-shaped ligand-binding site located within the bottom half of the LBD, extending from the C-terminal  $\alpha$ -helix (known as AF-2) to the  $\beta$ -sheet between helices 3 and 6. The presence of an additional helix 2' (Figure 2A), which is not seen in the steroid, retinoid, or thyroid receptor LBDs, may be responsible for the large volume of the PPAR $\gamma$  ligand-binding site.<sup>12,13</sup> Rosiglitazone binds in a U-shaped conformation, while occupying only 40% of the ligand-binding site. The TZD headgroup makes several specific hydrogen-bonding interactions with His<sup>449</sup>, Tyr<sup>473</sup>, His<sup>323</sup>, Ser<sup>289</sup>, and Gln<sup>286</sup> (Figure 4A). Tyr<sup>473</sup> lies on the C-terminal AF-2 helix, which is critical for transcriptional



**Figure 2.** Crystal structures of the apo-PPAR LBDs. The  $\alpha$ -helices are represented by tubes. Helix 2', helix 3, and the AF-2 helix are designated as H2', H3, and AF-2, respectively. The solvent-accessible ligand-binding site is indicated by the white shaded surface: A. PPAR $\gamma$  LBD; B. PPAR $\delta$  LBD.

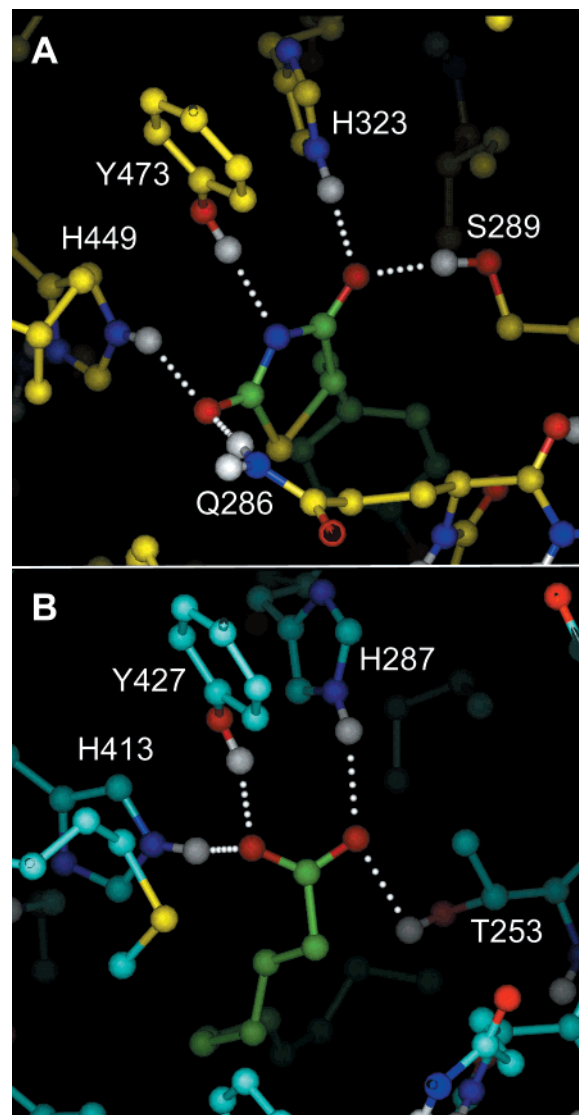




**Figure 3.** Crystal structure of the ternary complex of the PPAR $\gamma$  LBD with rosiglitazone and SRC1. Each PPAR $\gamma$  LBD of the homodimer is shown as a yellow worm. The side chains of the charge clamp residues Lys<sup>301</sup> and Glu<sup>471</sup> are colored orange. The SRC1 fragment containing the LxxLL motifs is shown in purple, but the linking peptide which showed poor electron density has been omitted. The TZD ligand is color coded by atom: A. side view of the complex, Lys<sup>301</sup> and Glu<sup>471</sup> are indicated; B. bottom view of the complex.

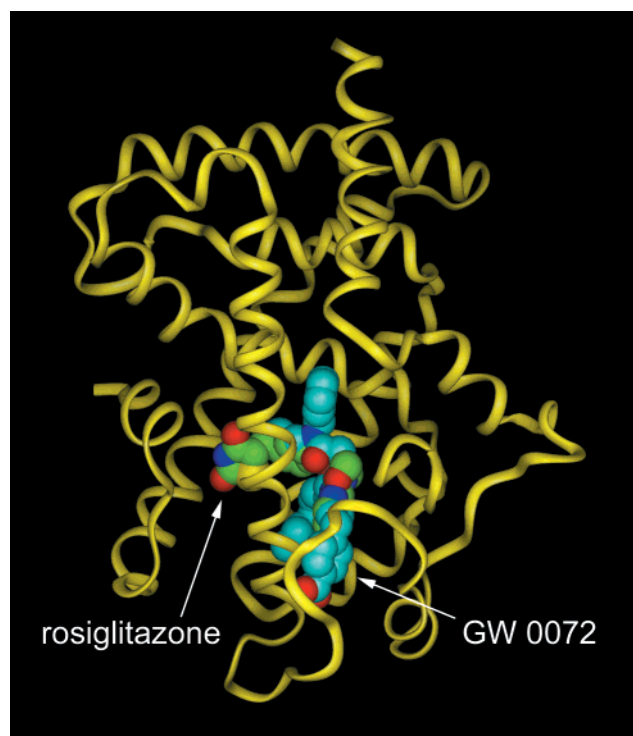
activation and provides the first example of a nuclear receptor ligand that interacts directly with a residue in this helix. Interestingly, in both the apo and ligand-bound structures of PPAR $\gamma$ , the AF-2 helix adopted a conformation similar to other ligand-bound nuclear receptors.<sup>193</sup> Although these structures do not provide clear evidence of a ligand-induced conformational change of the AF-2 helix, it is likely that interaction of the ligand with this helix locks the receptor in an activated state.

The ternary complex of PPAR $\gamma$  with rosiglitazone and SRC1 revealed the receptor crystallized as a homodimer bridged by a single molecule of the coactivator (Figure 3).<sup>12</sup> The homodimer is likely to use the same dimer interface as the physiologically relevant PPAR $\gamma$ /RXR heterodimer.<sup>23</sup> Each receptor in the dimer interacts with a short  $\alpha$ -helix from the SRC1 fragment through a 'charge clamp' formed by Glu<sup>471</sup> on AF-2 and Lys<sup>301</sup> on helix 3 (Figure 3A). Each  $\alpha$ -helix in the SRC1 fragment contains the peptide sequence LxxLL, where L = Leu and x = any amino acid. The charge clamp places the LxxLL motif into the optimal orientation to bury its Leu residues into a hydrophobic cleft on the surface of the



**Figure 4.** Conserved hydrogen-bonding interactions of PPAR agonists with the LBD. The key amino acids are indicated with their hydrogen bonds to the ligand drawn as white broken lines: A. TZD headgroup of rosiglitazone bound to PPAR $\gamma$ ; B. carboxylic acid headgroup of EPA bound to PPAR $\delta$ .

receptor (Figure 3B). Both LxxLL motifs make identical contacts with the receptor dimer, which may allow the multiple LxxLL motifs found in most coactivator proteins to mediate cooperative binding to nuclear receptor dimers. These structures revealed that binding of the PPAR ligand directly to the AF-2 helix stabilized the formation of the charge clamp on the surface of the receptor, which in turn is critical for recruitment of coactivator proteins to the receptor complex through their LxxLL motifs. Further support for this mechanism comes from the X-ray crystal structure of PPAR $\gamma$  bound to the partial agonist GW 0072.<sup>192</sup> In this structure (Figure 5), GW 0072 has a binding mode in which the ligand does not contact the AF-2 helix.<sup>192</sup> Although the backbone of the AF-2 helix was observed in the agonist-bound conformation, Tyr<sup>473</sup>, His<sup>323</sup>, and His<sup>449</sup> adopted side-chain conformations similar to those in the apo-PPAR $\gamma$  LBD crystal structure.<sup>192</sup> This suggests that in the GW 0072-bound receptor the charge clamp would not be stabilized through direct interactions with the ligand, which may account for the weak recruitment of



**Figure 5.** Partial agonist GW 0072 has a different binding mode to PPAR $\gamma$  compared to rosiglitazone. PPAR $\gamma$  LBD from the cocrystal with GW 0072 is shown as a yellow worm. GW 0072 is shown with its carbon atoms colored blue. Rosiglitazone was superimposed on the structure by alignment of the corresponding PPAR $\gamma$  LBD protein (not displayed). Rosiglitazone is shown with its carbon atoms colored green.

coactivator proteins.<sup>192</sup> Thus, it appears that the large binding pocket of PPAR $\gamma$  is not only capable of binding a number of structurally diverse ligands (Figure 5) but can also be exploited to identify ligands with altered binding characteristics and modified receptor pharmacology.

**PPAR $\gamma$  and Diabetes.** The most extensively studied therapeutic utility for PPAR $\gamma$  agonists has been in the treatment of type 2 diabetes.<sup>194</sup> TZD PPAR $\gamma$  agonists have been shown to enhance the sensitivity of target tissues to insulin and to reduce plasma glucose, lipid, and insulin levels in animal models of type 2 diabetes as well as in humans.<sup>195–199</sup> Troglitazone (Rezulin), pioglitazone (ACTOS), and rosiglitazone (Avandia) have been approved by the FDA and are currently marketed agents for the treatment of type 2 diabetes. These drugs represent an important advance in the treatment of this disease when used as monotherapy or in combination with existing therapies. Troglitazone has produced significant reductions in plasma glucose, HbA<sub>1c</sub>, insulin, and C-peptide levels, either alone or in combination with sulfonylureas or metformin in type 2 diabetics.<sup>200–202</sup> In addition, troglitazone therapy has been shown to have beneficial effects on serum lipids,<sup>203</sup> blood pressure, and cardiac output.<sup>204</sup> However, a significant number of diabetics fail to respond to troglitazone therapy, and concerns have been expressed about the potential for weight gain<sup>205,206</sup> or increased adipogenesis in bone marrow<sup>207</sup> that is seen with TZDs in rodents.

A much more serious issue with troglitazone is hepatotoxicity. Troglitazone has been linked to a number of cases of liver failure<sup>208</sup> that has resulted in its

withdrawal for use as monotherapy in newly diagnosed type 2 diabetics in the United States and withdrawal of the drug from the market in the United Kingdom. It is presently unclear whether the observed hepatotoxicity is mediated by PPAR $\gamma$  or by some other mechanism unique to troglitazone. Neither rosiglitazone nor pioglitazone has displayed the increased incidence of hepatic adverse events seen with troglitazone, suggesting that hepatotoxicity may not be a class effect of PPAR $\gamma$  agonists. Troglitazone has also been shown to activate the human pregnane X receptor (PXR) [NR1I2]<sup>11</sup> at the concentrations needed to activate PPAR $\gamma$ .<sup>209</sup> PXR is a recently isolated orphan member of the nuclear receptor gene family that was shown to be a key transcriptional regulator of hepatic cytochrome P450 3A4 (*CYP3A4*) gene expression.<sup>210–213</sup> Since *CYP3A4* is responsible for the oxidative metabolism of around 60% of all clinically used drugs, activation of PXR may be the molecular mechanism underlying the observed drug–drug interactions of troglitazone.<sup>214,215</sup> Troglitazone is also a substrate for *CYP3A4*, leading to formation of a potentially reactive quinone metabolite.<sup>216,217</sup> Thus, the cross-reactivity of troglitazone on PXR may lead to increased hepatic levels of the quinone metabolite in some patients. In addition, other known activators of PXR cause hepatomegaly in rats, suggesting that PXR may regulate additional genes involved in liver metabolism.<sup>210</sup> PXR is remarkably divergent across species, and troglitazone does not activate the mouse or rat receptor.<sup>209</sup> This may explain why hepatotoxicity was not seen in the rodent safety studies of the drug. While it remains to be proven that activation of PXR by troglitazone is responsible for the hepatotoxicity in humans, screening of new PPAR $\gamma$  agonists for selectivity against PXR should lead to the development of diabetes drugs with reduced propensity for P450 enzyme induction and drug–drug interactions.<sup>209</sup>

RXR agonists are also known to activate the PPAR/RXR heterodimer and can show additive activity with PPAR agonists in cell-based transactivation assays.<sup>23,218</sup> Interestingly, targetin (LGD1069) and LG100268, selective RXR agonists that had been developed primarily as anticancer drugs, have shown glucose- and lipid-lowering activity in rodents.<sup>218,219</sup> These effects are likely to be mediated through activation of the PPAR $\gamma$ /RXR and PPAR $\alpha$ /RXR heterodimers, although the activation of other RXR heterodimers cannot be ruled out. Notably, reports from human clinical trials indicate that these drugs may raise serum triglycerides<sup>220</sup> or lead to alterations in thyroid metabolism.<sup>221,222</sup> Thus, while it is still unclear that RXR agonists will be used as monotherapy for diabetes, they may be useful in combination therapy to increase the glucose- or lipid-lowering activity of various PPAR agonists.

The discovery that PPAR $\gamma$  is the molecular target of the antidiabetic TZDs has raised two apparent paradoxes. First, how can a receptor expressed mainly in adipose tissue improve insulin sensitivity and glucose utilization in skeletal muscle, the major insulin-sensing tissue? Several explanations have been proposed. Troglitazone has been shown to ameliorate insulin resistance and hyperglycemia in mice engineered to lack adipose tissue, suggesting that PPAR $\gamma$  action can occur by adipocyte-independent pathways.<sup>223</sup> Although PPAR $\gamma$

levels are 10–100-fold higher in adipose than in muscle or liver, the receptor is expressed in these latter tissues.<sup>131,224–226</sup> Thus, stimulation of PPAR $\gamma$  in these non-adipose tissues could contribute to altered gene expression and a reduction in insulin resistance and improved glucose disposal. PPAR $\gamma$  may also exert direct effects on genes involved in glucose homeostasis, although surprisingly little is known about this topic. One potential target gene for PPAR $\gamma$  that is central to improving muscle glucose disposal is the insulin-dependent glucose transporter *GLUT4*. TZD activation of PPAR $\gamma$  has been shown to increase the expression of this gene in adipocytes,<sup>227,228</sup> but direct regulation of its expression in muscle has not been reported. UCP2, a mitochondrial uncoupling protein related to UCP1, has been shown to be expressed in a number of tissues including skeletal muscle and adipose tissue and appears to function as an important modulator of energy usage.<sup>229</sup> Expression of UCP2 was up-regulated by TZD activation of PPAR $\gamma$  in cell lines derived from skeletal muscle, WAT, and brown adipose tissue (BAT).<sup>148,150</sup> Finally, PPAR $\gamma$  agonists may regulate the storage or release adipocyte-derived signaling factors that affect insulin sensitivity in muscle. Fatty acids are key mediators of this process. It is well-established that increased fatty acid concentrations decrease glucose metabolism in muscle.<sup>230</sup> While PPAR $\gamma$  agonists induce LPL, FATP-1, and ACS in adipose tissue, their expression is apparently unchanged in muscle.<sup>231</sup> This may allow for an increase in fatty acid clearance into adipose tissue with concomitant decrease in uptake of fatty acids into muscle, potentially improving insulin sensitivity.<sup>230</sup> In support of this hypothesis TZD treatment is reported to reduce the triglyceride content of muscle in diabetic rats.<sup>232</sup> The mechanism of these effects may be related to the observation that PPAR $\gamma$  regulates expression of the fatty acid transporter CD36,<sup>233</sup> which has been implicated in the control of insulin sensitivity in rats.<sup>234</sup> PPAR $\gamma$ -induced differentiation in WAT is also associated with a marked decrease in the levels of TNF $\alpha$  and leptin, two signaling molecules that are secreted by adipocytes.<sup>235,236</sup> Elevated levels of TNF $\alpha$  can cause insulin resistance,<sup>237</sup> and TZDs have been shown to block TNF $\alpha$ -induced insulin resistance in both cell culture<sup>238</sup> and animals.<sup>239</sup>

The second apparent paradox lies in the fact that obesity is a major risk factor in the development of type 2 diabetes. How then is a receptor that promotes adipogenesis still able to enhance insulin sensitivity? Evidence suggests that PPAR $\gamma$ -mediated differentiation of white adipocytes in rodents produces an increased number of small adipocytes while decreasing the number of large adipocytes.<sup>235,236</sup> These smaller adipose cells are usually more sensitive to insulin and would be expected to provide greater insulin-dependent glucose uptake. The smaller adipose cells should also have lower rates of lipolysis relative to larger adipose cells. Since high levels of free fatty acids have been linked to the induction of insulin resistance,<sup>230,240,241</sup> the decreased amount of circulating free fatty acid levels would be expected to have beneficial effects on insulin sensitivity. TZDs also induce the differentiation of BAT in rodents<sup>152</sup> and induce the expression of the mitochondrial uncoupling protein gene *UCP1* in human primary

adipocytes.<sup>242</sup> These effects may also contribute to increased energy consumption and decreased glucose and lipid levels.

Several polymorphisms have been identified in the human PPAR $\gamma$  gene. A rare Pro<sup>115</sup>Gln mutation in the PPAR $\gamma$ 2 N-terminal domain, resulting in a constitutively active receptor, was identified in four obese German individuals.<sup>243</sup> This phenotype was consistent with PPAR $\gamma$  playing a role in the regulation of fat storage in humans. A more common silent C/T-polymorphism in exon 6 was detected in a sample of a northern French population that was associated with higher plasma leptin levels in obese subjects but not body mass index (BMI).<sup>244</sup> A relatively common Pro<sup>12</sup>Ala mutation in the PPAR $\gamma$ 2-specific exon B has also been identified independently by two research groups.<sup>245,246</sup> Deeb found this mutation was associated with lower BMI, improved insulin sensitivity, and higher plasma HDLc concentrations.<sup>246</sup> The association with insulin sensitivity disappeared when corrected for BMI, indicating that the mutation primarily affects body weight. From a population of obese Finns, two women who were severely overweight were found to carry both the exon B and exon 6 polymorphisms.<sup>247</sup> However, other reports have found no association of the Pro<sup>12</sup>Ala mutation with BMI<sup>248</sup> and insulin sensitivity.<sup>249</sup> Subsequent reports<sup>250–253</sup> have failed to resolve this apparent conflict, underscoring the influence of environmental factors and the difficulty in determination of these phenotypes in humans.

In contrast to the human genetic studies, recent reports<sup>254–256</sup> of the phenotype of the PPAR $\gamma$  null mouse have shed additional light on the role of PPAR $\gamma$  in adipocyte function and insulin resistance. The PPAR $\gamma$  null mutation was embryonic lethal around day 10 of gestation due to a defect in placental development.<sup>254,255</sup> As expected, PPAR $\gamma^{-/-}$  embryonic stem cells and fibroblasts failed to undergo differentiation to a mature adipocyte phenotype in cell culture.<sup>255,256</sup> Using a placental rescue technique Barak showed that PPAR $\gamma$  was not essential for further development of the mice.<sup>254</sup> A single mouse that was brought to full-term lacked adipose tissue and showed extreme metabolic defects, similar to the human condition of lipodystrophy.<sup>257</sup> These results confirmed the essential role of PPAR $\gamma$  in the formation of mature adipocytes and its important role in lipid and glucose homeostasis mediated through adipose tissue. Notably, the heterozygous PPAR $\gamma^{+/-}$  mice develop normally, have a normal number and size of adipocytes, and show no metabolic defects. In an elegant experiment, Kubota reported that upon challenge with a high-fat diet, the PPAR $\gamma^{+/-}$  mice were partially protected from weight gain and the development of insulin resistance when compared to their wild-type litter mates.<sup>255</sup> This surprising result was explained by the observation that the adipocytes of the PPAR $\gamma^{+/-}$  mice were less hypertrophic, and the circulating leptin levels were 2-fold higher than in the wild-type mice. UCP expression was also 20% higher in the BAT of the PPAR $\gamma^{+/-}$  mice. The differences were consistent with the decreased food intake and higher basal metabolic rate that was observed in the PPAR $\gamma^{+/-}$  mice. These results raise the intriguing possibility of a dichotomy in PPAR $\gamma$  function with respect to insulin



sensitization.<sup>255</sup> On the one hand, PPAR $\gamma$  activation may promote adipocyte differentiation and the formation of small adipocytes leading to increased insulin sensitivity. On the other hand, PPAR $\gamma$  activation may cause hyperplasia of preexisting adipocytes and suppression of leptin production,<sup>258</sup> with a consequent decrease in insulin sensitivity. Thus, it is possible that what was once a thrifty gene, which evolved to aid the storage and release of fat during cycles of feast and famine, may now predispose modern humans to obesity and type 2 diabetes in our time of relative caloric excess.<sup>259</sup>

**PPAR $\gamma$  and Dyslipidemia.** It is well-established that PPAR $\gamma$  agonists decrease plasma levels of triglycerides, cholesterol, and nonesterified fatty acids in various animal models of dyslipidemia.<sup>155</sup> These lipid changes are also seen in humans, although the effects are less consistent. There appear to be some minor differences among the three marketed PPAR $\gamma$  agonists, although not enough clinical data are available yet on rosiglitazone and pioglitazone to afford a clear comparison. Troglitazone has shown a consistent decrease in plasma triglycerides (13–32%) and free fatty acids (up to 22%), while effects on cholesterol metabolism are variable, with some clinical trials reporting an increase in both HDLc (up to 16%) and LDLc (up to 13%) while others report no change in cholesterol levels.<sup>202,260</sup> In clinical trials, rosiglitazone has shown variable results on triglyceride lowering but appears to lower free fatty acids while raising both HDLc and LDLc.<sup>261</sup> Pioglitazone also lowers triglycerides and raises HDLc; however, LDLc does not appear to increase upon pioglitazone treatment, with levels being no different than placebo.<sup>262</sup> The origin of the observed differences in lipid effects among these TZDs remains unclear. On the basis of the current data, PPAR $\gamma$  agonists would appear to have some utility in lowering triglycerides and free fatty acids. However, no clinical trials with selective PPAR $\gamma$  agonists have been conducted on patient populations that are hyperlipidemic but not diabetic, so the pharmacological profile of these agents in a strictly dyslipidemic setting is unknown.

**PPAR $\gamma$  and Hypertension.** Hypertension is increasingly recognized as a complex metabolic and cardiovascular disorder. The pathogenesis of the disease involves improper regulation of blood pressure, insulin sensitivity, vascular function, and lipid metabolism.<sup>263</sup> TZDs have been shown to decrease blood pressure in a number of animal models, including Dahl S rats,<sup>264</sup> obese Zucker rats,<sup>265,266</sup> spontaneously hypertensive rats,<sup>267</sup> Watanabe rabbits,<sup>268</sup> and obese insulin-resistant rhesus monkeys.<sup>269</sup> Pioglitazone was reported to decrease blood pressure in the one-kidney, one-clip Sprague–Dawley rat, an animal model of hypertension not associated with insulin resistance.<sup>270–271</sup> This suggests that the ability of PPAR $\gamma$  agonists to prevent hypertension may be independent of their ability to improve insulin resistance. Troglitazone has been shown to reduce blood pressure in nondiabetic and diabetic humans<sup>202</sup> and improve overall cardiac output and stroke volume without an increase in cardiac mass.<sup>204</sup> The mechanism by which PPAR $\gamma$  agonists exert their antihypertensive effects is not well-understood. TZDs have been shown to inhibit growth factor-induced vascular smooth muscle

cell proliferation in cell culture<sup>264,272</sup> and in animals,<sup>273,274</sup> potentially through PPAR $\gamma$ -mediated inhibition of MAP kinase.<sup>275,276</sup> PPAR $\gamma$  agonists may also decrease blood pressure by affecting vascular contractility.<sup>277</sup> TZDs have been reported to modulate the production of several key factors involved in maintenance of vascular tone, including type-C natriuretic peptide,<sup>278</sup> endothelin,<sup>278,279</sup> and PAI-1.<sup>280,281</sup> Finally, TZDs have been shown to modulate currents in a number of ion channels. Troglitazone, pioglitazone, and rosiglitazone all suppress the voltage-gated (L-type) Ca<sup>2+</sup> current in rat aortic myocytes.<sup>282,283</sup> However, the rank order of potency for Ca<sup>2+</sup> channel inhibition does not correlate with potency for PPAR $\gamma$  activation, which suggests that the effect of these compounds on ion channels may be mediated through PPAR $\gamma$ -independent mechanisms.

**PPAR $\gamma$  and Inflammation.** The potential role of PPAR $\gamma$  in regulation of inflammatory processes was suggested by studies in adipose tissue, in which a general antagonism was seen between PPAR $\gamma$  and the proinflammatory cytokine TNF $\alpha$ . PPAR $\gamma$  agonists have been shown to reduce the expression level of TNF $\alpha$  in the adipose tissue of obese rats and to block the inhibitory effects of TNF $\alpha$  on insulin signaling<sup>238</sup> and TNF $\alpha$ -induced glycerol and free fatty acid release.<sup>284</sup> Monocytes and macrophages have a well-established involvement in inflammatory processes, including vascular wall inflammation and atherogenesis, through production of nitric oxide (NO) and inflammatory cytokines such as TNF $\alpha$ , IL-1, and IL-6. Chinetti<sup>285</sup> found that PPAR $\gamma$  expression was induced upon differentiation of human monocytes into macrophages. Activation of PPAR $\gamma$  by rosiglitazone or 15d-PGJ<sub>2</sub> resulted in induction of apoptosis due to interference with the NF- $\kappa$ B signaling pathway. Activation of PPAR $\gamma$  in macrophages has been shown to inhibit MMP-9 activity.<sup>286</sup> Treatment of activated macrophages with PPAR $\gamma$  agonists resulted in a change in cell morphology and suppression of NO production.<sup>287</sup> PPAR $\gamma$  agonists, including certain NSAIDs, block the production of inflammatory cytokines in human monocytes treated with phorbol myristate acetate.<sup>288</sup> However, it is important to point out that in both of these latter studies, the antiinflammatory activity seen with rosiglitazone occurred at concentrations considerably higher than the  $K_d$  value for binding to PPAR $\gamma$  or the concentration needed to elicit adipogenesis and insulin sensitization. Recent studies have demonstrated that activation of PPAR $\gamma$  by either 15d-PGJ<sub>2</sub> or rosiglitazone in colonic cell lines attenuated inflammatory cytokine gene expression through inhibition of NF- $\kappa$ B activation.<sup>289</sup> Furthermore, both troglitazone and rosiglitazone reduced colonic inflammation in an established murine model of colitis, suggesting that PPAR $\gamma$  agonists may have therapeutic potential in the treatment of inflammatory bowel disease.<sup>289</sup>

A recent report<sup>233</sup> also proposed that PPAR $\gamma$  might have proinflammatory or proatherogenic activity. PPAR $\gamma$  protein is abundantly expressed in foam cells of human atherosclerotic lesions.<sup>133</sup> Foam cells are cholesterol-laden macrophages that can accumulate below the arterial wall endothelium and contribute to atherosclerotic plaque formation. The conversion of macrophages to foam cells involves the internalization of oxidized

LDL (oxLDL) particles. This internalization does not occur through the LDL receptor but rather through lipid transporters such as CD36, SR-A, and others.<sup>290</sup> Treatment of HL60 monocytic leukemia cells with a combination of 15d-PGJ<sub>2</sub> and the RXR agonist LG100268 caused induction of macrophage markers and increased expression of CD36 and uptake of oxLDL.<sup>233</sup> The induction of CD36 suggests the possibility of an oxLDL–PPAR $\gamma$ –CD36 positive feedback loop.<sup>233</sup> However, it is important to point out that this induction of CD36 expression required the combination of a PPAR $\gamma$  agonist and an RXR agonist—treatment with a PPAR $\gamma$  agonist alone could not induce CD36. Thus it is also possible that RXR agonists contribute to foam cell formation through activation of other signaling pathways.

The results presented to date portray a somewhat conflicting story on the consequences of PPAR $\gamma$  activation in the context of inflammation and atherogenesis.<sup>291</sup> One difficulty in interpreting the results of the aforementioned experiments is that many investigators have employed the naturally occurring activator 15d-PGJ<sub>2</sub>, which also has cellular activity independent of PPAR $\gamma$ . In addition most of the data has been generated in cell culture, where results are often difficult to extrapolate to human physiology. The current clinical data in humans suggests that troglitazone-mediated activation of PPAR $\gamma$  does not promote atherogenesis. Troglitazone has been shown to decrease serum PAI-1 levels in some insulin-resistant patient groups<sup>292</sup> and to reduce atherosclerosis by decreasing the intima and media thickness in carotid arteries of type 2 diabetics.<sup>293</sup> Thus, further studies are necessary to resolve these questions and explore the potential of PPAR $\gamma$  agonists in the treatment of inflammation and atherosclerosis.

**PPAR $\gamma$  and Cancer.** Activation of PPAR $\gamma$  by TZDs has been shown to trigger cell cycle arrest in logarithmically growing NIH-3T3 fibroblasts and in malignantly transformed adipogenic HIB-1B cells.<sup>294</sup> The effects of PPAR $\gamma$  activation on differentiation and cell cycle regulation have prompted a number of investigations into the utility of PPAR $\gamma$  agonists for the treatment of cancerous tumors. Activation of PPAR $\gamma$  with pioglitazone in primary human liposarcoma cells has been shown to result in differentiation and withdrawal from the cell cycle.<sup>295</sup> Recently troglitazone was shown to induce terminal adipocytic differentiation in humans with advanced liposarcoma.<sup>296</sup> Since current chemotherapy regimens for inoperable liposarcomas are reported to give complete response rates in less than 10% of patients, PPAR $\gamma$  agonists have potential as chemotherapeutic agents for treatment of liposarcomas. PPAR $\gamma$  is also expressed in human primary and metastatic breast tumors.<sup>297</sup> Treatment of cultured breast cancer cells with troglitazone resulted in a reduction in growth rate and changes in cell morphology and gene expression representative of a more differentiated state. Interestingly, upon treatment with troglitazone for several days, the cells retained substantially reduced capacity for growth after cessation of drug treatment. Troglitazone has also been shown to inhibit tumor growth of MCF-7 cells in BNX triple-immunodeficient mice.<sup>298</sup> The tyrosine-based PPAR $\gamma$  agonist GW 7845 has been shown to significantly reduce tumor incidence, tumor size, and average tumor burden in the NMU-

induced rat model of mammary carcinoma.<sup>299</sup> Additive, but not synergistic, effects were seen with GW 7845 and a suboptimal dose of tamoxifen in this model. Consistent with these effects, expression of PPAR $\gamma$  is decreased in tumorigenic rodent mammary glands.<sup>300</sup>

PPAR $\gamma$  is expressed in human colon tumors and colon cancer cell lines.<sup>301,302</sup> Activation of PPAR $\gamma$  in these cell lines with PPAR $\gamma$  agonists caused inhibition of cell growth and cell cycle arrest, with morphologic changes consistent with colonic differentiation. Human colorectal cancer cells implanted in nude mice were shown to grow more slowly in mice treated with troglitazone, with a 50% reduction in tumor volume.<sup>301</sup> In addition, somatic mutations in PPAR $\gamma$  have been found in a subset of human primary colorectal carcinomas.<sup>303</sup> Each mutation led to a loss of receptor function when exposed to troglitazone or 15d-PGJ<sub>2</sub>. These data suggest that activation of PPAR $\gamma$  might have a therapeutic benefit in the treatment of colorectal cancer. However, two groups have independently reported that PPAR $\gamma$  agonists increase the size and frequency of polyps in *APC*<sup>Min/+</sup> mice, an animal model genetically predisposed to intestinal neoplasia.<sup>304,305</sup> These mice are a model of the human genetic disease familial adenomatous polyposis coli (FAP), which is characterized by polyps and invasive neoplasia in the small and large intestines. In addition to FAP, the human *APC* gene is often mutated in sporadic colon cancer. It is not clear whether the increased polyp formation induced by activation of PPAR $\gamma$  in this murine model resulted in invasive carcinoma, as no clear examples were documented in either study. In addition, neither study found an increase in polyps in genetically nonsusceptible mice. Therefore, the potential for increased incidence of colon tumors in humans may be confined to those at risk of developing APC-negative cancers. Due to the discrepancy in these results,<sup>301,303–305</sup> however, further study of the role of PPAR $\gamma$  in colon carcinogenesis is needed.

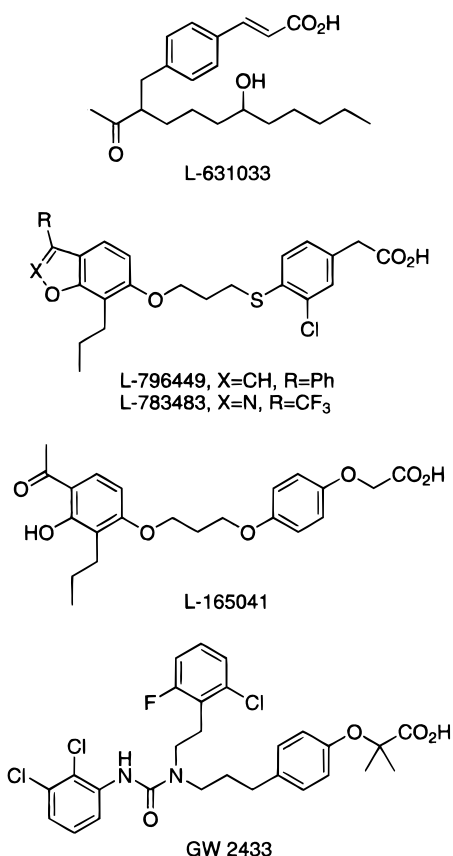
Despite the apparent dichotomy in results with colon cells, these initial studies on the effects of PPAR $\gamma$  activation on cell cycle control have revealed the potential for using PPAR $\gamma$  as a therapeutic target in certain forms of cancer. Although extrapolation from cell culture and rodent studies to humans is complicated and often inaccurate, the antimitotic and differentiation-promoting abilities of PPAR $\gamma$  agonists warrant further human study. The fact that clinical use of troglitazone in diabetics has shown no overt toxicity in either colonic or breast tissue to date provides support for additional studies on the use of PPAR $\gamma$  agonists, either alone or in combination with more conventional chemotherapeutic agents, in the treatment of human cancers.

## PPAR $\delta$

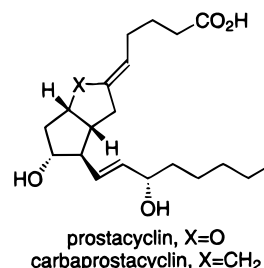
PPAR $\delta$  has been cloned from a number of species and initially given a variety of names. The receptor was first reported as PPAR $\beta$  in *Xenopus laevis*<sup>50</sup> and NUC1 in humans.<sup>306</sup> Subsequently, the receptor was cloned from mice as PPAR $\delta$ ,<sup>15</sup> NUC1,<sup>307</sup> and FAAR.<sup>308</sup> Today, the generally agreed upon name for this receptor is PPAR $\delta$ . The human and rodent receptors are about 90% identical in the LBD, while the frog receptor shows somewhat lower sequence identity (72%). Human PPAR $\delta$  has been mapped to chromosome 6p21.1–p21.2. PPAR $\delta$  mRNA is

ubiquitously expressed in adult rat tissues, but often at lower levels than either PPAR $\alpha$  or PPAR $\gamma$ .<sup>54</sup> A study with human tissues showed that PPAR $\delta$  was present in liver, intestine, kidney, abdominal adipose, and skeletal muscle, tissues that are all involved in aspects of lipid metabolism.<sup>55</sup>

**Synthetic Ligands.** Unlike the other subtypes, there are no known drugs that have been identified as working through PPAR $\delta$ .<sup>48</sup> Thus, part of the challenge in determining the function of PPAR $\delta$  has been the identification of potent and selective ligands for use as chemical tools.<sup>49</sup> It is important to note that all of the ligands published to date either have low affinity for PPAR $\delta$  or lack selectivity over the other PPARs (Table 1). The Merck group, which originally cloned NUC1, has reported a series of synthetic ligands, such as L-631033, as weak activators of the receptor.<sup>309</sup> These compounds resemble fatty acids with a rigidifying ring in the middle of the chain, reminiscent of some eicosanoids. Recently this group has reported a more potent series of PPAR $\delta$  agonists.<sup>45</sup> The established leukotriene antagonist<sup>310</sup> L-165041 was identified through random screening as an activator of human PPAR $\delta$ . This compound shows 10-fold selectivity for human PPAR $\delta$  over human PPAR $\alpha$  and PPAR $\gamma$  but is only weakly active on the murine PPAR $\delta$  with little selectivity over murine PPAR $\gamma$  (Table 1). The phenylacetic acid derivatives L-796449 and L-783483 were potent dual agonists of murine PPAR $\delta$  and PPAR $\gamma$  but activated all three human PPAR subtypes. We have also identified a series of potent PPAR $\delta$  ligands from a biased three-component library of ureidofibrates.<sup>44</sup> GW 2433 was a high-affinity ligand for human PPAR $\delta$  and a dual activator of PPAR $\delta$  and PPAR $\alpha$  in cell-based transactivation assays (Table 1).

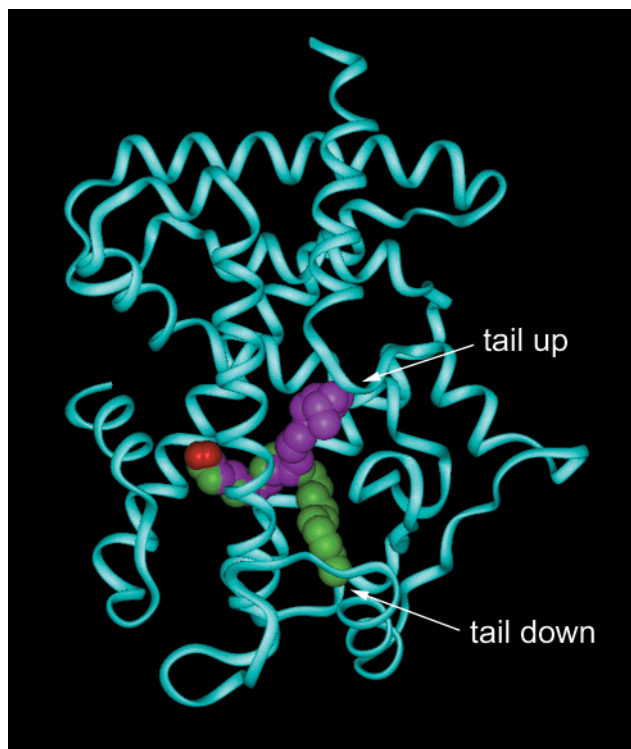


**Natural Ligands.** In common with the other subtypes, PPAR $\delta$  is a receptor for naturally occurring fatty acids. A systematic study of the binding of fatty acids to PPAR $\delta$  found that both saturated and unsaturated fatty acids bound to the receptor.<sup>14</sup> This binding profile was intermediate between that of PPAR $\alpha$  and PPAR $\gamma$ . Among the polyunsaturated fatty acids, dihomog $\gamma$ -linolenic acid, arachidonic acid, and EPA bound with affinities in the low micromolar range.<sup>14,73</sup> In a search for natural ligands of PPAR $\delta$ , methyl palmitate was isolated from acetone extracts of pancreatic tissue.<sup>311</sup> The fact that the free acid was not isolated as a PPAR $\delta$  ligand is surprising, but the authors noted that the pancreas has high levels of enzymatic activity for conversion of fatty acids into their methyl esters. Another group<sup>308</sup> found that palmitic acid and the metabolically stable 2-bromopalmitic acid were activators of murine PPAR $\delta$ . Several eicosanoids have been shown to activate PPAR $\delta$  including PGA<sub>1</sub> and PGD<sub>2</sub>.<sup>75</sup> The semisynthetic prostaglandin carbaprostacyclin was reported to be one of the most efficacious activators of PPAR $\delta$  at micromolar concentrations.<sup>73</sup> Unfortunately, the naturally occurring prostacyclin (PGI<sub>2</sub>) is too unstable to be assayed.



**Protein Structure.** The crystal structure of the apo-PPAR $\delta$  LBD has been determined by molecular replacement, using the PPAR $\gamma$  crystal structure as a model (Figure 2B).<sup>14</sup> The overall structure of the PPAR $\delta$  LBD was very similar to that of PPAR $\gamma$  including the general size of the ligand-binding pocket. However, differences in the residues lining the ligand-binding sites result in subtle changes to the shape of the pocket (Figure 2). A second structure was determined as a cocrystal with the natural ligand EPA (Figure 6).<sup>14</sup> Interestingly, EPA was observed bound to the receptor in two distinct conformations. The acid group and the first eight carbons adopted the same orientation in both conformations, while the tail (carbons 9–20) either pointed upward toward the upper lipophilic arm of the pocket or pointed downward into the third leg of the pocket. The ability of EPA to adopt multiple low-energy conformations within the receptor may be the molecular basis of the promiscuous binding of fatty acids to the PPARs and suggests that the need to sense multiple low-affinity hormones provided the evolutionary drive for the large ligand-binding site.<sup>14</sup> The carboxyl group of EPA was held in place by a network of hydrogen bonds from the side chains of His<sup>413</sup>, Tyr<sup>427</sup>, His<sup>287</sup>, and Thr<sup>253</sup> (Figure 4B). Tyr<sup>427</sup> is part of the AF-2 helix, and the hydrogen bond formed with the ligand is likely to stabilize the conformation that facilitates coactivator recruitment. The same network of hydrogen bonds was observed in the ternary complex of PPAR $\gamma$  with rosiglitazone and SRC1 (Figure 4A).<sup>12</sup> The remarkable conservation of this





**Figure 6.** Crystal structure of EPA bound to the PPAR $\delta$  LBD. The PPAR $\delta$  LBD is shown as a blue worm. The two conformations of EPA observed in the crystal structure are shown in purple (tail up) and in green (tail down).

network of hydrogen bonds suggests that it must be crucial for ligand-mediated activation of these receptors. The cocrystal of PPAR $\delta$  with GW 2433 revealed that this fibrate ligand also formed the same network hydrogen-bonding interactions with the AF-2 helix.<sup>14</sup> Interestingly, this molecule filled all the three legs of the Y-shaped pocket, which may explain why it bound with such high affinity to the receptor.

**PPAR $\delta$  and Dyslipidemia.** It is likely that PPAR $\delta$  is involved in lipid homeostasis because, like the other two subtypes, fatty acids and fatty acid metabolites activate the receptor. Amri showed that 3TC-C2 fibroblasts transfected with a PPAR $\delta$  expression vector became responsive to fatty acids.<sup>308</sup> Specifically, two early markers of adipocyte differentiation, the adipocyte lipid-binding protein and the adipocyte fatty acid transporter (FAT/CD36), were induced. These effects may be due, in part, to PPAR $\delta$  induction of PPAR $\gamma$  expression in the cells.<sup>312</sup> Pharmacological activation of PPAR $\delta$  does not appear to have the same adipogenic potential as PPAR $\gamma$ .<sup>313</sup> The molecular basis for these differences was recently explored. Using a series of chimeric receptors created by domain swaps between PPAR $\delta$  and PPAR $\gamma$ , Castillo reported that the N-terminal domain of PPAR $\gamma$  was largely responsible for adipogenic potential of the receptor.<sup>16</sup> Thus, a chimeric receptor containing the PPAR $\delta$  LBD and DBD fused to the PPAR $\gamma$  N-terminal domain could induce adipogenesis when stimulated by 2-bromopalmitic acid. These results suggest that although the highly conserved LBD and DBD are essential for the function of the receptor, it is the poorly conserved N-terminus that dictates the adipogenic potential of the receptor.

The first proposed pharmacological role for PPAR $\delta$  has been the regulation of cholesterol homeostasis.

Plasma cholesterol was raised in *db/db* mice treated with 30 mg/kg L-165041.<sup>45</sup> Although this compound shows only modest selectivity over PPAR $\gamma$  on the murine receptors (Table 1), the pharmacological effect was ascribed to activation of PPAR $\delta$  since neither serum triglycerides nor glucose were lowered at this dose. Interestingly, in this same model a series of dual activators of PPAR $\delta$  and PPAR $\gamma$  showed glucose-lowering activity, suggesting that activation of PPAR $\delta$  did not inhibit insulin sensitization through PPAR $\gamma$ .<sup>45</sup>

**PPAR $\delta$  and Fertility.** The role of PPAR $\delta$  has been studied in blastocyte implantation and decidualization in mice.<sup>314</sup> PPAR $\delta$  was the only subtype expressed in the uterus during the implantation period in mice. Expression was induced in the stroma surrounding the implanting blastocyst and became localized in the decidual zone post-implantation. This expression pattern was mirrored by the expression of prostacyclin synthase. Cyclooxygenase-2 (COX2) null mice show defects in implantation and decidualization. Since COX2 is involved in the synthesis of prostacyclin, it was hypothesized that *COX2*<sup>-/-</sup> mice may be deficient in endogenous activators of PPAR $\delta$ .<sup>314</sup> In support of this hypothesis, PPAR $\delta$  activators such as carbaprostacyclin or L-165041 in combination with 9-*cis*-retinoic acid were shown to restore implantation in the *COX2*<sup>-/-</sup> mice. Thus, prostacyclin or its metabolites may be regulators of embryo implantation through activation of PPAR $\delta$ .

**PPAR $\delta$  and Cancer.** A recent report has suggested that NSAIDs mediate their antitumorigenic activity in colorectal cancer in part through PPAR $\delta$ .<sup>315</sup> High micromolar concentrations of the NSAIDs sulindac and indomethacin suppressed PPAR $\delta$  activity in a transactivation assay. In colon cancer cells, sulindac-induced apoptosis was partially inhibited by adenoviral overexpression of PPAR $\delta$ . The authors inferred that NSAIDs reduce the incidence of intestinal tumors by inhibiting PPAR $\delta$  function and promoting apoptosis.<sup>315</sup> However, this proposal has yet to be tested using more potent PPAR $\delta$  ligands.

## Summary and Perspectives

It is nearly a decade since the first PPAR subtype was reported as an orphan member of the nuclear receptor gene family.<sup>9</sup> Few could have predicted in 1990 that the PPARs would emerge as therapeutic targets with widespread impact in the treatment of human metabolic diseases. The initial characterization of these orphan receptors focused on their roles as lipid-regulated transcription factors and the regulation of hepatic peroxisomal enzyme expression.<sup>316</sup> The role of these receptors in the induction of peroxisome proliferation is now understood to be species specific, with only rodents showing this response to activators of the PPAR $\alpha$  subtype.<sup>87</sup> By contrast, all three PPARs appear to function as hormone receptors for dietary fatty acids and certain eicosanoids across multiple species and in many metabolically active tissues.<sup>317</sup>

A full appreciation of the therapeutic potential of these receptors required the identification of synthetic ligands that were drugs with formerly unknown mechanisms of action. The fibrate and glitazone drugs were developed by a succession of pharmaceutical companies over a period of 40 years using empirical medicinal

chemistry and rodent pharmacology. Although their cellular targets were unknown, these drugs had been successfully employed in the treatment of hypertriglyceridemia or type 2 diabetes in humans. The demonstration that PPAR $\alpha$  and PPAR $\gamma$  were the receptors through which the fibrate<sup>71</sup> and glitazone<sup>160</sup> drugs mediate their biological activity has led to a renaissance in nuclear receptor research to develop drugs for diabetes and cardiovascular disease.<sup>318,319</sup> Knowledge of the molecular targets for the fibrates and glitazones has enabled medicinal chemists to synthesize a new generation of drugs that have been optimized for activity against the human PPARs. Several of these drugs are currently in clinical development. From our own laboratories, the selective PPAR $\gamma$  agonist GI 262570 is currently in phase III clinical trials for the treatment of type 2 diabetes.<sup>320</sup> Compounds with dual PPAR $\gamma$  and PPAR $\alpha$  activity, which may combine the benefits of insulin sensitization and lipid lowering into a single drug, are also being investigated.<sup>41,161,320</sup> These dual agonists may be particularly effective in those diabetic patients that have the additional risk factor of high serum triglycerides.<sup>321</sup>

Even with the rapid progress that has occurred in characterization of these orphan receptors, several intriguing questions remain unanswered. What are the natural hormones and physiological functions of the PPARs? The accumulated evidence suggests that the PPARs are receptors for fatty acids and eicosanoids. However, it is still unclear how many of the currently identified naturally occurring ligands are true physiological hormones. To qualify for this designation, it needs to be demonstrated that a candidate ligand is found at sufficient concentrations in the correct tissues and cells and that its associated biology is physiologically relevant. For example, since PPAR $\gamma$  plays a critical role in regulation of lipid and glucose homeostasis through its role in adipocyte differentiation, we might expect that its natural hormone(s) would be associated with the epidemiology of type 2 diabetes.<sup>5</sup> What is the pharmacology of PPAR $\delta$  ligands? While PPAR $\delta$  is likely to be another fatty acid receptor,<sup>14</sup> its broad expression pattern gives few clues to its role in lipid metabolism or its other functions. This receptor will be a true test of the reverse endocrinology paradigm,<sup>49</sup> since PPAR $\delta$  does not appear to be a receptor for any class of known drugs. The results of the study of PPAR $\delta$ -selective ligands, especially in nonrodent animal models, are eagerly awaited. Do PPAR agonists have therapeutic utility beyond the metabolic disorders of diabetes and cardiovascular disease? Diets with a high content of polyunsaturated fatty acids have been claimed to show benefit in several chronic inflammatory diseases such as eczema, psoriasis, and arthritis.<sup>322,323</sup> In addition, the debate on the role of dietary saturated fat as an environmental factor in the development of cancer has raged for many years. The chemoprotective effects of polyunsaturated fatty acids in animal studies are supported by human epidemiological data on the incidence of cancer in Westernized nations; however these effects have yet to be confirmed in prospective human clinical trials.<sup>324,325</sup> The evaluation of synthetic PPAR agonists as drug therapies for nonmetabolic diseases will ultimately be required to answer this question.

The PPARs are now some of the most intensely studied members of the nuclear receptor gene family. Basic research on these receptors has increased our understanding of the molecular mechanism of hormone action, the role of fatty acids as hormones, and the role of adipose tissue as an endocrine organ. The promiscuity of the PPARs for their ligands and the availability of high-resolution X-ray structures has led to the development of new concepts about molecular recognition and the evolution of low-affinity hormone receptors. Throughout this research several apparent paradoxes in hormone function have come and gone, yet much remains to be explored. New technologies that enable transcript profiling through differential gene expression<sup>326</sup> will undoubtedly increase our knowledge of the mode of action of the PPARs through the identification of their key target genes. Thus, as we enter the 21st century, we may have the potential to develop PPAR drugs with increased efficacy and safety through understanding the molecular basis of their biological action. These drugs may be particularly useful in the growing population of prediabetic patients<sup>2</sup> that exhibit multiple risk factors for cardiovascular disease, such as impaired glucose tolerance and hypertriglyceridemia.<sup>91,321</sup> The success of these drugs will provide an acid test of the value of predictive molecular medicine over empirical drug discovery.

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## Biographies

**Timothy M. Willson** received his Ph.D. degree from the University of Southampton and completed his postdoctoral training at the University of Illinois at Urbana–Champaign. He joined the Glaxo Research Institute in 1990 and is currently Head of Nuclear Receptor Target Discovery at Glaxo Wellcome. His research focuses on the orphan members of the nuclear receptor gene family as targets for drug discovery.

**Peter J. Brown** received his Ph.D. degree from the University of Sheffield. Following postdoctoral training at Indiana University, he joined the Cardiovascular Chemistry Group at Bristol Myers. In 1989 he moved to the Glaxo Research Institute (now Glaxo Wellcome), where his current research involves the application of combinatorial chemistry to the discovery of orphan nuclear receptor ligands.

**Daniel D. Sternbach** received his Ph.D. degree from Brandeis University and completed his postdoctoral training at the ETH–Zurich and Harvard University. He joined the chemistry faculty at Duke University in 1979 and moved to the Glaxo Research Institute (now Glaxo Wellcome) in 1986. His research in medicinal chemistry has involved anticancer therapy and more recently metabolic diseases.

**Brad R. Henke** received his Ph.D. degree from the University of Illinois at Urbana–Champaign and completed his postdoctoral training at the University of California, Berkeley. He joined the Glaxo Research Institute in 1991 and is currently a Senior Research Investigator at Glaxo Wellcome. He led the chemistry team that identified the human PPAR $\gamma$  agonist GI 262570. His current research interests lie in the areas of metabolic and musculoskeletal diseases.

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