The Structure−Activity Relationship between Peroxisome Proliferator-Activated Receptor γ Agonism and the Antihyperglycemic Activity of Thiazolidinediones

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Introduction. Resistance of peripheral tissues to the action of insulin is a characteristic feature of human obesity and non-insulin-dependent diabetes (NIDDM).[1] A number of prospective studies have shown that the development of insulin resistance is an early event in the natural progression of the disease.[2] Thus, drugs that reverse the onset of insulin resistance fulfill a major unmet medical need for the treatment of NIDDM.[3] Thiazolidinediones are a class of oral insulin-sensitizing agents that improve glucose utilization without stimulating insulin release. They significantly reduce glucose, lipid, and insulin levels in rodent models of NIDDM and obesity,[4] and recent clinical data supports their efficacy in obese diabetic patients.[5] The original lead for the identification of this class of compounds was clofibrate (**1a**, Figure 1), which possesses both antilipidemic and weak antihyperglycemic activity in humans.[6] Scientists at Takeda, through screening analogs of **1a** for increased antihyperglycemic activity in diabetic mice, identified the acid **2** and subsequently the thiazolidinedione **3** (Ciglitazone).[7] Although the molecular mechanism of action remained unknown, analog synthesis and *in vivo* screening over a period of 15 years led to the identification of thiazolidinediones with increased potency.[8] Examples of thiazolidinediones that have progressed to clinical evaluation are the Pfizer compound Englitazone (**4**),[11] the Takeda compound Pioglitazone (**5**),[9] and the Beecham compound BRL 49653 (**6**).[10]

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear receptor superfamily of ligand-activated transcription factors. Three subtypes of PPARs have been cloned from mouse and human: PPARα, PPARγ, and PPARδ (also known as NUCI).[12] The PPARs are believed to play a physiological role in the regulation of lipid metabolism. They can be activated by high concentrations of fatty acids (FA) and have been shown to regulate the expression levels of FA binding proteins or enzymes involved in FA oxidation.[13] Since the natural ligand for these receptors has yet to be identified, they are classified as "orphan" receptors. We recently identified the thiazolidinedione **6** as first high-affinity ligand for PPARγ, a receptor subtype selectively expressed in adipocytes and shown to induce adipocyte differentiation.[14] This report details the structure−activity relationship between compounds that activate PPARγ *in vitro* and their reported antihyperglycemic activity in genetically obese and diabetic mice *in vivo*.

Results. A series of compounds (**1b**−**11**) was chosen from the literature that had been previously tested in rodent models of diabetes.[4,7,9−11] Clofibric acid (**1b**), the active form of clofibrate (**1a**),[15] was purchased from Sigma. Compounds **2**−**11** were synthesized by the literature methods.[7,9−11] The compounds were tested in a transient transfection assay in CV-1 cells for their ability to activate PPARs. To allow comparison of the relative transcriptional activity of the three receptors on the same target gene and to prevent endogenous receptors from complicating the interpretation of results, an established receptor chimera system was used.[14] The ligand binding domains of mouse PPARα, PPARγ, and PPARδ were each fused to the DNA-binding domain of the yeast transcription factor GAL4. CV-1 cells were transiently transfected with expression vectors for the respective PPAR chimera together with a reporter construct containing five copies of the GAL4 upstream activator element driving expression of secreted placental alkaline phosphatase (SPAP). Weak activity on PPARα was observed for only **1b** and **2**, and no activity was seen on PPARδ for any of the compounds (Table 1). By contrast, it was found that all the compounds (**1b**−**11**) were efficacious activators of PPARγ, showing 25−30-fold induction of reporter gene activity at the highest dose tested (data not shown). The potency of compounds **1b**−**11** as judged by the EC_{50} for PPARγ activity, was structure dependent and spanned a wide dose range from millimolar (for **1b**) to nanomolar (for **10**).

In addition to screening for functional activity in cells, compounds **1b**−**11** were tested for their ability to bind directly to PPARγ *in vitro*. [^{3}H]-**6** was prepared by tritiodebromination of **12** (Scheme 1). [^{3}H]-**6** was isolated with a specific activity of 41 Ci/mmol. The ligand binding domain of PPARγ was expressed in *Escherichia coli* as a fusion protein with glutathione *S*-transferase.[14] In competition binding assays, compounds **1b**−**11** showed specific displacement of [^{3}H]-**6** from the PPARγ ligand binding domain fusion protein. Thus, compounds **1b**−**11** are PPARγ agonists since they bind directly to the receptor and activate transcription in a comparable dose range.

Discussion. The results in Table 1 show that thiazolidinediones **3**−**11** were selective PPARγ agonists, whereas acids **1b** and **2**, although less potent, showed comparable activity on PPARα and PPARγ. None of the compounds demonstrated measurable activity on PPARδ.[16] Compounds **3** and **5**−**10** have been reported to show antihyperglycemic activity in genetically obese C57 Bl/6 *ob*/*ob* mice,[10] an animal model of NIDDM that is insulin resistant, hyperinsulinemic, and glucose intolerant. Table 1 shows the reported minimum effective dose (MED) for antihyperglycemic activity using an oral glucose tolerance test following dosing of **3** and **5**−**10** in the diet for 8 d.[10,17] Comparison of the EC_{50} for activation of PPARγ with the MED for anti-

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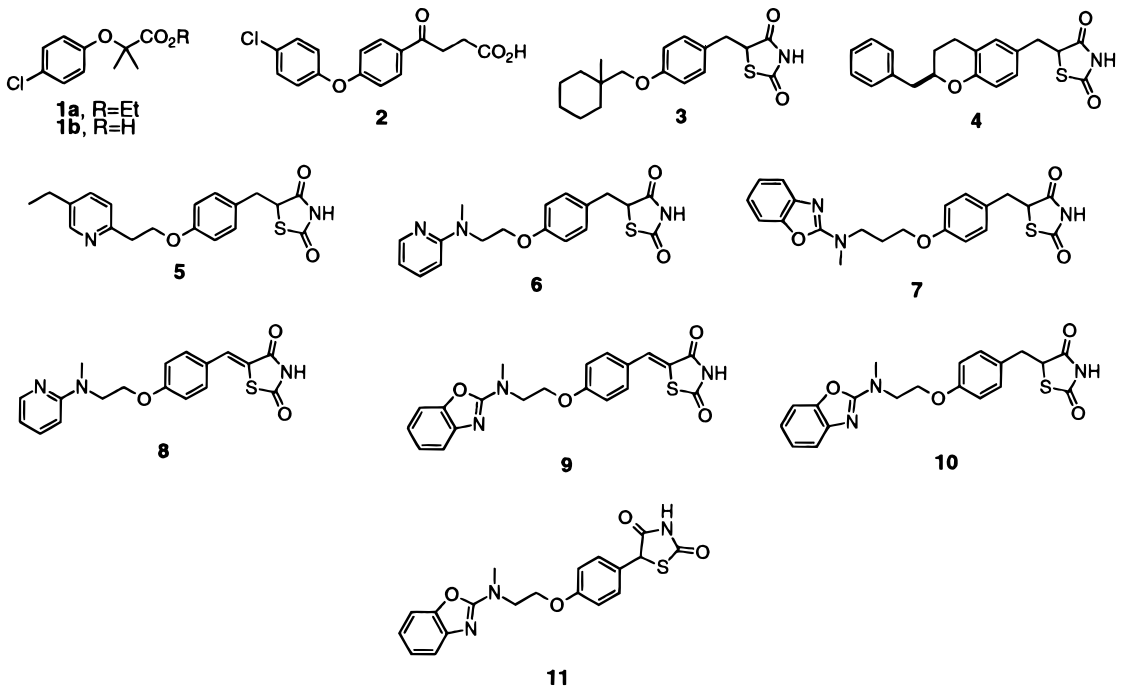


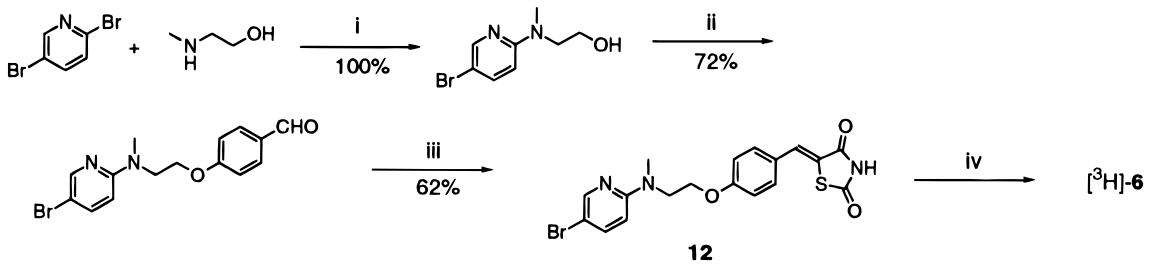
Figure 1.

Table 1. *In Vitro* and *in Vivo* Activity of Compounds (**1b**−**11**)

	transactivation[a]			binding:[b]	*in vivo*[c]
no.	PPARα EC_{50} (μM)	PPARγ EC_{50} (μM)	PPARδ EC_{50} (μM)	PPARγ (% displacement)	MED (μmol kg^{-1})
1b	51 ± 2	600 ± 200	ia	56 ± 2	
2	80 ± 20	110 ± 20	ia	86 ± 3	
3	ia	3.0 ± 0.7	ia	81 ± 4	3000
4	ia	13 ± 2	ia	89 ± 1	
5	ia	0.69 ± 0.08	ia	90 ± 1	300[d]
6	ia	0.060 ± 0.004	ia	89 ± 2	3
7	ia	1.0 ± 0.7	ia	79 ± 6	300
8	ia	0.19 ± 0.03	ia	85 ± 6	300
9	ia	0.14 ± 0.04	ia	80 ± 6	300
10	ia	0.013 ± 0.003	ia	93 ± 1	3
11	ia	10 ± 2	ia	83 ± 5	ia at 1000

[a] GAL4-PPAR chimeric expression constructs were prepared as described previously (ref 14). Reporter plasmid UAS-tk-SPAP was generated by insertion of five copies of a GAL4 DNA-binding element into pG12-tk-SPAP (ref 22). CV-1 cells were transfected with the relevant receptor expression plasmid, reporter construct and β-galactosidase expression plasmid (pCH110, Pharmacia) as described previously (ref 14). After 16 h, the medium was exchanged to DME medium supplemented with 10% delipidated fetal calf serum and the test compound at the appropriate concentration. After an additional 24 h, cell extracts were prepared and assayed for alkaline phosphatase activity and β-galactosidase activity. Alkaline phosphatase activity was corrected for transfection efficiency using the β-galactosidase activity as internal standard (ref 23). EC_{50} equals the concentration of compound required to induce 50% of the maximum alkaline phosphatase activity ± standard error, $n = 3$. ia = inactive at 10^{-3} M (**1** and **2**) or 10^{-4} M (**3**−**11**). [b] Competition binding was performed with bacterial extracts containing the glutathione *S*-transferase-PPARγ ligand binding domain as described previously (ref 14). Percent displacement equals the percent of [^{3}H]-**6** (10^{-7} M) competitively displaced by the test compound at a concentration of 10^{-4} M (except **1b** and **2** were at 10^{-3} M) in triplicate ± standard error. [c] MED = minimum effective dose for significant reduction in blood glucose in *ob*/*ob* mice (from ref 10a). [d] See comment in ref 17.

Scheme 1[a]



[a] Reagents: (i) neat, 150 °C, 18 h; (ii) NaH, 4-fluorobenzaldehyde, DMF, 80 °C, 18 h; (iii) 2,4-thiazolidinedione, piperidine, EtOH, reflux, 5 h; (iv) $^3H_2$(g), 10% Pd−C, DMF, 5 h.

hyperglycemic activity revealed a significant correlation ($r^2 = 0.92$, $P < 0.0005$, Figure 2). Thus, for compounds **3** and **5**−**10**, both the relative and absolute rank order potency for activation of PPARγ *in vitro* mirrored the *in vivo* antihyperglycemic activity in diabetic *ob*/*ob* mice.

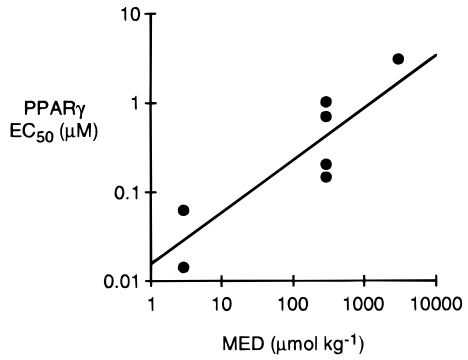


Figure 2. Correlation of *in vitro* PPARγ activity with *in vivo* antihyperglycemic activity in *ob*/*ob* mice for compounds **3** and **5**−**10**. *In vitro* PPARγ EC_{50} from Table 1. *In vivo* minimum effective dose (MED) for antihyperglycemic activity from ref 10a; $r^2 = 0.92$, $P < 0.0005$.

Table 1 shows that compounds **1b**, **2**, **4**, and **11** also demonstrated PPARγ agonist activity. Compound **11** was reported to be inactive at 1000 μmol kg^{-1} in *ob*/*ob* mice,[10a] and Figure 2 predicts that a dose of >10 000 μmol kg^{-1} would be required to show *in vivo* activity. Thus, the *in vitro* PPARγ activity of **11** is consistent with the reported *in vivo* data. Englitazone (**4**) was reported to be active in *ob*/*ob* mice at doses comparable to ciglitazone (**3**);[11] however, differences in dosing regimes (diet vs gavage) and duration of study (8 vs 4 d) did not allow inclusion of this compound in the correlation.[10,11] Finally, the activities of **1b** and **2** have not been reported in *ob*/*ob* mice, although **2** was reported to lower blood glucose in diabetic KK-A^y mice at high doses (0.2% of diet for 4 d).[7a]

Within a single compound class that is known to have acceptable pharmacokinetic properties, it is likely that differences in *in vivo* potency reflect changes in the pharmacodynamic properties of the molecules. Thus, the correlation between *in vivo* potency of compounds **3** and **5**−**10** and the measured *in vitro* activity implicates PPARγ as the molecular target for the antidiabetic effects of thiazolidinediones.[18] PPARγ is predominantly expressed in adipose tissue,[19] and it was recently shown that PPARγ, through ectopic expression, can act as a master regulator of adipocyte differentiation in preadipocyte and multipotential cell lines.[14,20] Since skeletal muscle is the predominant insulin-sensitive tissue in the body, accounting for >80% of the insulin-regulated glucose disposal, an increase in glucose disposal in adipocytes alone is unlikely to account for the dramatic antihyperglycemic activity of the thiazolidinediones. Thus, the mechanism by which activation of PPARγ-regulated genes in adipocytes reverses insulin resistance remains to be discovered.[21]

Conclusion. The structure−activity relationship for PPARγ agonist activity *in vitro* accurately predicts the *in vivo* antihyperglycemic activity of thiazolidinediones in diabetic mice. The ability to rapidly screen compounds against this target in radioligand competition-binding and functional transactivation assays should allow the rapid development of more potent and selective antidiabetic agents.

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Supporting Information Available: Experimental details and spectral and analytical data for the preparation of the radioligand ([^{3}H]-**6**) (2 pages). Ordering information is given on any current masthead page.

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(17) The MED for compound **5** is reported as 300 μmol kg^{-1} in ref 10a and 200 μmol kg^{-1} in ref 10b. The higher value has been used in Table 1 and in Figure 2. If the lower value is used, the correlation in Figure 2 shows $r^2 = 0.91$, $P < 0.0005$.

(18) A reviewer has noted that a number of structurally diverse thiazolidinediones of varying potencies exist which were not included in our analysis. Therefore caution should be exercised in extending our results to cover these other classes of compounds.

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