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Sulfonylureas and Glinides Exhibit Peroxisome Proliferator-Activated Receptor γ Activity: A Combined Virtual Screening and Biological Assay Approach

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ABSTRACT

Most drugs currently employed in the treatment of type 2 diabetes either target the sulfonylurea receptor stimulating insulin release (sulfonylureas, glinides), or target the peroxisome proliferator-activated receptor (PPARγ) improving insulin resistance (thiazolidinediones). Our work shows that sulfonylureas and glinides additionally bind to PPARγ and exhibit PPARγ agonistic activity. This activity was predicted in silico by virtual screening and confirmed in vitro in a binding assay, a transactivation assay, and by measuring the expression of PPARγ target genes. Among the measured compounds, gliquidone and glipizide (two sulfonylureas), as well as nateglinide (a glinide), exhibit PPARγ agonistic activity at concentrations comparable with those reached under pharmacological treatment. The most active of these compounds, gliquidone, is shown to be as potent as pioglitazone at inducing PPARγ target gene expression. This dual mode of action of sulfonylureas and glinides may open new perspectives for the molecular pharmacology of antidiabetic drugs, because it provides evidence that drugs can be designed that target both the sulfonylurea receptor and PPARγ. Targeting both receptors could increase pancreatic insulin secretion and improve insulin resistance. Glinides, sulfonylureas, and other acidified sulfonamides may be promising leads in the development of new PPARγ agonists. In addition, we provide a unified concept of the PPARγ binding ability of seemingly disparate compound classes.

Poor eating habits and sedentary lifestyle have led to an increasing prevalence of metabolic disorders, such as type 2 diabetes mellitus, which is attaining epidemic proportions (World Health Organization, 2002). Primary prevention by dietary adjustments and increased exercise remains the most desirable and cost-effective strategy. Nevertheless, pharmacotherapeutic intervention to prevent severe immediate and long-term consequences of type 2 diabetes is often unavoidable.

The peroxisome proliferator-activated receptors (PPARs) are involved in the regulation of lipid and glucose metabolism (Willson et al., 2001). They are ligand-dependent transcription factors that contain an N-terminal activation domain, a DNA-binding domain, and a ligand-binding domain (Renaud and Moras, 2000). PPARs activate target genes by binding to response elements located within regulatory regions of these target genes (Laudet and Gronemeyer, 2002). Accessory proteins, termed coactivators and corepressors, associate with DNA-bound PPARs and modulate the expression of target genes through chromatin structure modifications and recruitment of the transcription machinery (Hermanson et al., 2002). Three subclasses of PPARs are known, called α, γ, and δ, that are coded by different genes, exhibit tissue-specific expression patterns, and are associated with various functions. Of these, PPARγ is expressed mostly in adipose tissue, where it is essential in adipocyte differentiation and controls the storage of fatty acids, increasing triglyceride synthesis and storage within adipocytes. In addition, there is strong evidence that PPARγ regulates glucose homeostasis (Willson

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ABBREVIATIONS: PPAR, peroxisome proliferator-activated receptor; SUR1, sulfonylurea receptor 1; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; C_{max}, mean maximal plasma concentration; FK614, 3-(2,4-dichlorobenzyl)-2-methyl-*N*-(pentylsulfonyl)-3*H*-benzimidazole-5-carboxamide; aP2, adipocyte fatty acid-binding protein gene; GLUT4, glucose transporter-4 gene.

et al., 2000, 2001; Wang and Tafuri, 2003; Rangwala and Lazar, 2004). Activation of PPARγ improves insulin resistance, and therefore PPARγ is an established molecular target for the treatment of type 2 diabetes (Perfetti and D'Amico, 2005; Staels and Fruchart, 2005).

For PPARγ, several unsaturated fatty acids have been proposed as natural ligands, in particular prostaglandins such as 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (Ferry et al., 2001), nitrolinoleic acids such as 10-nitrolinoleic acid (Schopfer et al., 2005), and putative metabolites of docosahexaenoic acid such as 4-hydroxydocosahexaenoic acid (Yamamoto et al., 2005). A few synthetic PPARγ agonists are approved drugs [e.g., rosiglitazone, pioglitazone, which are members of the glitazone (thiazolidinedione) class (Willson et al., 2001)] or are under development as antidiabetics [e.g., tesaglitazar (Ericsson et al., 2004) and muraglitazar (Cox, 2005)]. See Fig. 1 for chemical structures. All PPARγ agonists in clinical use or development (in fact, most known PPARγ agonists) are either thiazolidinediones or carboxylic acids (Martin et al., 2005). Many drug therapies targeting PPARγ have their disadvantages [e.g., the liver toxicity of glitazones (Hug et al., 2004), weight gain, fluid retention, enhanced adipogenesis, and cardiac hypertrophy (Picard and Auwerx, 2002)]. The development of an otherwise promising PPARγ ligand and drug candidate, farglitazar (see Fig. 1), had to be discontinued in phase III because of the emergence of edema (Parker, 2002; Shi et al., 2005). Therefore, demand is increasing for new PPARγ ligands, and compound classes other than carboxylic acids or thiazolidinediones could be of special interest.

The goal of the present study was to identify new PPARγ agonists among known drugs and biologically active compounds by combining virtual screening with experimental verification in biological assays. This strategy provides a detailed model of ligand-receptor complexes, together with an experimental confirmation of ligand-receptor binding and the consequent biological activity. We followed a three-step approach. First, a virtual screening search on two large databases of drugs and biologically active compounds allowed us to identify a few glinides and sulfonylureas as promising PPARγ ligands and prompted us to concentrate on these two drug classes, screening several more members thereof. Most of these compounds showed good affinities to PPARγ in silico. In a second step, we found that selected sulfonylureas and glinides bind to PPARγ and enhance PPARγ-mediated gene expression in vitro. In a third step, encouraged by these results, we screened in silico a few new compounds related to the sulfonylureas (i.e., *N*-acylsulfonamides), resulting in the prediction that they would bind PPARγ with relatively high binding affinities.

Sulfonylureas and glinides are hypoglycemic drugs in clinical use for the treatment of type 2 diabetes, by virtue of their insulin secretagogue properties. These compounds bind to the sulfonylurea receptor SUR1 on the membrane of β-cells, triggering the closure of the nearby potassium channel, which in turns leads the β-cell to increase insulin secretion (Farret et al., 2005). Our discovery that some insulin secretagogue drugs activate PPARγ has attractive implications for the pharmacological treatment of type 2 diabetes. Moreover, sulfonylureas and *N*-acylsulfonamides are new classes of PPARγ agonists.

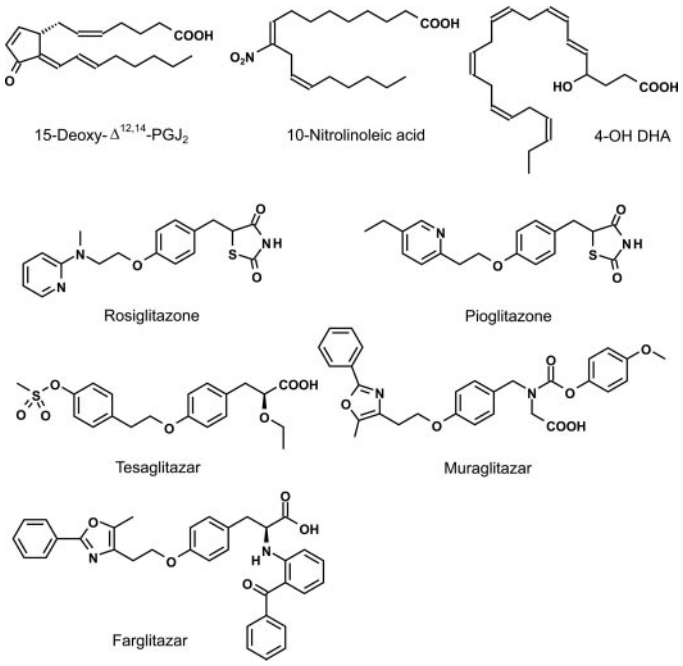

Fig. 1. Structures of some endogenous and synthetic PPARγ agonists. 4-OH DHA, 4-hydroxydocosahexaenoic acid.

Materials and Methods

Virtual Screening Database. The TheraSTrat AG inhouse database (Hug et al., 2003; Gut and Bagatto, 2005) contains most marketed drugs and many of their metabolites (approximately 8000 compounds). The freely available Chembank database contains approximately 6000 bioactive compounds (http://chembank.broad.harvard.edu).

Ligand Docking. Each compound was docked into the PPARγ binding site using the AutoDock 3.0.5 software (Morris et al., 1998). AutoDock finds several low-energy arrangements ("poses") of a given flexible ligand into a given receptor assumed to be rigid. For each pose, a pK_i value is calculated. The PPARγ three-dimensional structure was obtained from Protein Data Bank entry 1FM9. This is a 2.1-Å resolution crystal structure of the heterodimer of the human retinoid X receptor α and PPARγ ligand binding domains, bound to 9 *cis*-retinoic acid and farglitazar, respectively, together with coactivator peptides (Gampe et al., 2000). The PPARγ-farglitazar complex was imported in MOE (Chemical Computing Group Inc., Montreal, QC, Canada) where hydrogens were added and energy-minimized. The resulting structure without farglitazar was imported in AutoDock, where the protonation state of acidic and basic groups was adjusted (His323 and His449 were protonated), and partial charges were assigned. The protonation state of ligands to be docked was adjusted to the species assumed predominant at physiological pH. In particular, carboxylic acid, thiazolidinedione, sulfonylurea, and *N*-acylsulfonamide moieties were deprotonated. Partial charges were assigned according to the MMFF94x force field (MOE). For method verification and calibration, we docked to PPARγ a set of 121 carboxylic acids that are PPARγ agonists with known experimental binding affinities, a collection detailed in our earlier work (Rücker et al., 2006). For the compounds whose best pose showed both carboxylate oxygen atoms within 2 Å of the corresponding atoms of farglitazar in the X-ray structure (83% of the total), the pK_i calculated by AutoDock and the experimental pK_i correlated with $r^2 = 0.6$ (slope and intercept of the linear regression were 0.9 and 3.5, respectively). Because in this test AutoDock consistently overestimated experimental pK_i values, all calculated pK_i values given in the following are linearly rescaled using the above numbers for slope and inter-

cept. Among the many poses returned by AutoDock for each compound, we selected as best the one assigned the highest pK_i value, provided it had a hydrogen bond to Tyr473 and at least two further hydrogen bonds to His323, His449, or Ser289. In all PPARγ-ligand complexes with known X-ray structures, such hydrogen bonds seem to be essential for binding (Nolte et al., 1998; Gampe et al., 2000; Cronet et al., 2001; Xu et al., 2001; Sauerberg et al., 2002), and the hydrogen bond to Tyr473 was proposed to play a vital role for PPARγ coactivator recruitment (Cronet et al., 2001), which justify the constraints above.

Reagents and Plasmids. Standard cell culture and transfection reagents were purchased from Invitrogen (Carlsbad, CA). Charcoal-stripped delipidated FBS was purchased from Sigma-Aldrich (Buchs, Switzerland). Gliquidone was purchased from Apin Chemicals (Oxon, UK); glipizide, glimepiride, repaglinide, and linoleic acid were purchased from Sigma-Aldrich; nateglinide was purchased from Toronto Research Chemicals Inc. (North York, ON, Canada); and mitiglinide and pioglitazone were purchased from Molekula Ltd. (Dorset, UK). The 3xPPRE-Luc vector was kindly provided by Ron Evans (The Salk Institute for Biological Studies, San Diego, CA). The expression vector encoding human PPARγ was provided by Walter Wahli (Centre Integratif De Genomique, Lausanne, Switzerland).

Ligand Binding Assay. The Green PolarScreen PPAR Competitor Assay (Invitrogen) was used according to the manufacturer's instructions. This cell-free assay is based on purified recombinant human PPARγ ligand-binding domain and a selective fluorescent PPARγ ligand. The complex between the ligand and the ligand-binding domain exhibits high fluorescence polarization, which is lost upon ligand displacement by nonlabeled competitors. Millipolarization values for different competitors were determined in a ViewLux reader (PerkinElmer Life and Analytical Sciences, Boston, MA) by measuring the fluorescence intensities of the S (parallel to excitation) and P (perpendicular to excitation) channels, and background fluorescence of the assay buffer was subtracted. Relative specific activity (Ar) was determined by scaling measured values according to $Ar = (V - B)/(C - B)$, where V is the value measured, and B and C are the median millipolarization values of 0% and 100% control wells. IC_{50} values (concentrations at which 50% of the fluorescent ligand is displaced) and pIC_{50} values (negative decadic logarithm of IC_{50}) were determined for test compounds by measuring Ar values for a series of concentrations. A four-parametric sigmoidal curve was fitted to each data set using DataFactory (BioFocus DPI, Allschwil, Switzerland) or Data Analysis Toolbox (Elsevier MDL, San Ramon, CA), and the IC_{50} value was determined from the fitted curve.

Transactivation Assay. CV-1 cells were cultured in DMEM (4500 mg/l glucose), supplemented with 10% FBS and 50 U of penicillin/streptomycin. Three days before transfection, cells were sterol-depleted by exchanging the culture medium to DMEM/Ham's F12 medium without Phenol Red, supplemented with 10% charcoal-stripped FBS and 50 U of penicillin/streptomycin. Cells were plated in a 96-well dish with a density of 5×10^5 cells/ml (100 μl per well). DNA transfection was carried out in OptiMEM I (Invitrogen) without Phenol Red using Lipofectamine (Invitrogen). Each well received 8 ng of expression vector, 20 ng of reporter vector, and 60 ng of β-galactosidase vector. Twenty-four hours after transfection, drugs dissolved in dimethyl sulfoxide were added in DMEM/Ham's F12 without Phenol Red, supplemented with 10% charcoal-stripped delipidated FBS (Sigma-Aldrich), and 50 U of penicillin/streptomycin. Sixteen hours after addition of drugs, cells were lysed in chloramphenicol acetyltransferase assay lysis buffer (Catalys AG/Promega, Wallisellen, Switzerland). Supernatants were analyzed for luciferase activity by addition of luciferase reagent (Catalys AG/Promega). Background normalization was carried out by measuring β-galactosidase activity as described previously (Iniguez-Lluhi et al., 1997). EC_{50} values (concentrations at which 50% of the maximal gene expression is induced) and pEC_{50} values (negative decadic logarithm of EC_{50}) were determined for test compounds by measuring the increase of PPARγ target gene expression induced at different concentrations. A four-parametric sigmoidal curve was fitted to each data set using Prism software from GraphPad Software (San Diego, CA), and the EC_{50} value was determined from the fitted curve. Experiments were performed in quadruplicate, and error bars represent S.D.

Measurement of PPARγ Target Gene Expression. 3T3-L1 fibroblasts were purchased from European Collection of Cell Cultures (Porton Down, Wiltshire, UK). They were amplified in DMEM/10% calf serum and subsequently seeded into six-well plates. Two days after the cells reached confluence, the medium was changed to DMEM/10% fetal calf serum containing 0.5 M 3-isobutyl-1-methylxanthine, 2 μg/ml insulin (Actrapid), and 0.5 μM dexamethasone, to which the experimental compounds were added. Two days after induction of the cells, the medium was changed to DMEM/10% fetal calf serum containing 2 μg/ml insulin, to which the experimental compounds were freshly added. Compounds were tested at the following concentrations: rosiglitazone, 1 μM; pioglitazone, 10 μM; gliquidone, 10 μM; glipizide, 100 and 200 μM; nateglinide, 50 and 200 μM; and repaglinide, 50, 100, and 200 μM. The cells were harvested in TRIzol (Invitrogen, Breda, the Netherlands) 5 days after induction, and RNA was isolated by the standard procedure. RNA (1 μg) was used for cDNA synthesis using iScript (Bio-Rad Laboratories, Veenendaal, the Netherlands). cDNA was amplified with Platinum *Taq* polymerase using SYBR green on a Biorad MyiQ cycler. Specificity of the amplification was verified by melt curve analysis and evaluation of the amplification efficiency. Subsequently, expression of the genes of interest was normalized using cyclophilin as housekeeping gene.

The following primers were used: aP2: forward, AAGAAGTGGGAGTGGGCTTT; reverse, AATCCCCATTTACGCTGATG; GLUT4: forward, GGAAGGAAAAGGGCTATGCTG; reverse, TGAGGAACCGTCCAAGAATGA; cyclophilin: forward, TGTCTTTGGAACTTTGTCTGCAA; cyclophilin-reverse, CAGACGCCACTGTCGCTTT; adiponectin: forward, GCAGAGATGGCACTCCTGGA; reverse, CCCTTCAGCTCCTGTCATTCC.

Results

In a first step, all compounds in the TheraSTrat and Chembank databases were docked to PPARγ. Among them, repaglinide (a carboxylic acid belonging to the glinide group of drugs), sulfadimidine (an acidified sulfonamide), and glimepiride (a sulfonylurea) were thereby assigned relatively high binding affinities (Scarsi et al., 2005). In a second step of virtual screening, we focused on these compound classes, docking several more members thereof, as well as structurally related compounds.

Several Glinides and Sulfonylureas Are Docked to PPARγ with a High Binding Affinity. Figure 2, A to C, depict repaglinide, nateglinide, and mitiglinide, respectively, docked into PPARγ and superimposed to the farglitazar complex X-ray structure (for structures, see Fig. 3). The predicted bound conformations to PPARγ are similar to that of farglitazar. The carboxylate group of the glinides superimposes well with that of farglitazar, forming hydrogen bonds with residues His323, His449, Ser289, and Tyr473 of PPARγ, as farglitazar does. Repaglinide forms several hydrophobic contacts in the large apolar cavity (Fig. 2A, bottom left) that in the case of farglitazar is occupied by its long hydrophobic tail. Nateglinide and mitiglinide fit well the smaller hydrophobic cavity (Fig. 2, B and C, bottom right) that in the farglitazar complex is occupied by the benzoylphenyl group.

Repaglinide, mitiglinide, nateglinide, and meglitinide are predicted to bind PPARγ with pK_i values of 7.2, 6.3, 5.9, and 5.0, respectively. The smaller molecules mitiglinide, nateg-

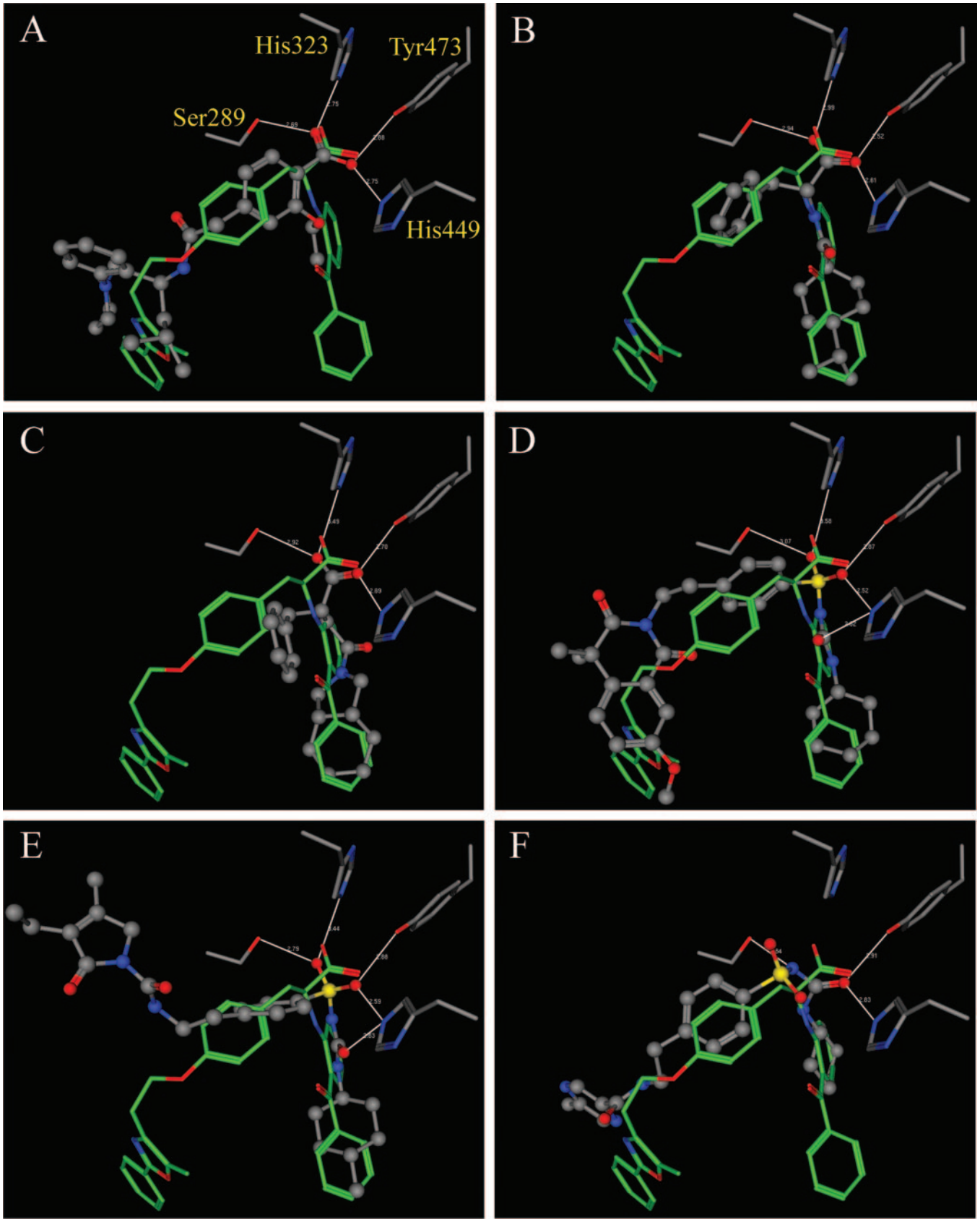

Fig. 2. Three-dimensional structures of three glinides and three sulfonylureas docked to PPARγ. Repaglinide (A), nateglinide (B), mitiglinide (C), gliquidone (D), glimepiride (E), and glipizide (F) (gray ball and stick models) docked to PPARγ and superimposed to the farglitazar complex X-ray structure (green stick model). Of PPARγ, only the side chains of His323, His449, Ser289, and Tyr473 are shown (gray stick models). Compounds were docked into the PPARγ binding site using the AutoDock software. For each ligand, the figure reports the best docked pose. For details on pose selection, see *Materials and Methods*.

linide, and meglitinide (molecular mass, <350 Da) bind at a lower affinity compared with the larger repaglinide (molecular mass, 453 Da), because the latter forms more favorable contacts between the hydrophobic cavities of the PPARγ binding site and its large hydrophobic moieties.

Figure 2, D to F, show gliquidone, glimepiride, and glipizide, respectively, docked into PPARγ and superimposed to the farglitazar complex X-ray structure (for structures, see Fig. 3). The predicted binding mode of gliquidone and glimepiride in the polar part of the binding site exhibits interesting similarities to that of farglitazar. In analogy to the carboxylate oxygens of farglitazar, the sulfonamide oxygen atoms point toward the pocket built by the side chains of His323, His449, Ser289, and Tyr473 and form hydrogen bonds to the H donor atoms in these residues. It is noteworthy that in the case of gliquidone and glimepiride, there are two alternatives for H-bond formation to His449, either to a sulfonyl oxygen or to the urea oxygen atom. Glimepiride deviates considerably in the lower part of the binding cavity from the bound conformation of farglitazar. This is not unexpected, because this part of the binding cavity is wide, allowing some conformational freedom for the ligand (Nolte

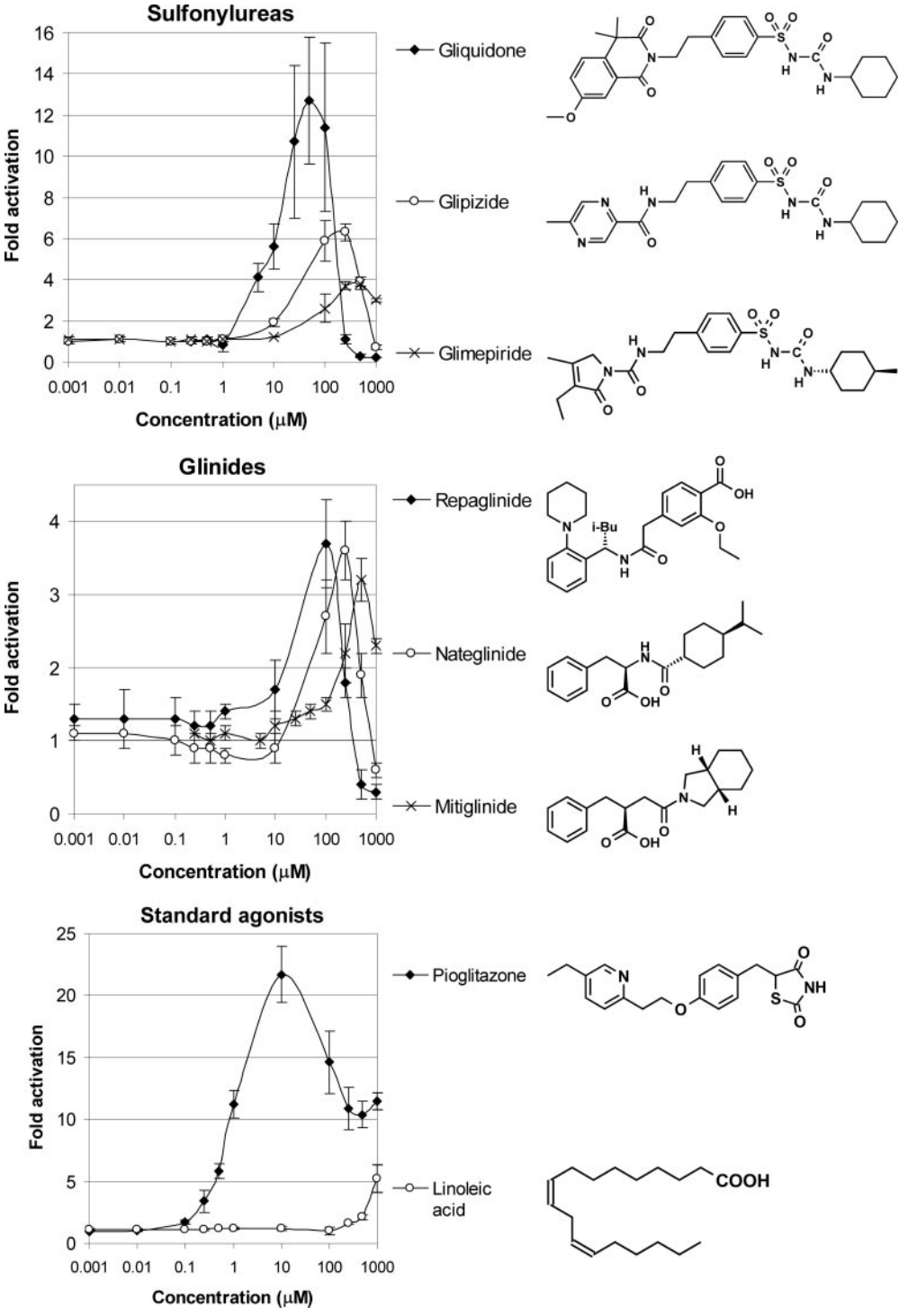

Fig. 3. Induction of PPARγ-mediated gene expression. The effects of three sulfonylureas (gliquidone, glipizide, and glimepiride; top graph), three glinides (repaglinide, nateglinide, and mitiglinide; middle graph), and two standard agonists (pioglitazone and linoleic acid; bottom graph) on PPARγ-dependent transactivation were assayed in CV-1 cells. Cells were transfected with expression vector encoding human PPARγ, 3xPPRE-Luc reporter vector, and β-galactosidase vector as described under *Materials and Methods*. After 24 h of transfection, cells were treated for 16 h with indicated concentrations of each compound. Luciferase activity was normalized by β-galactosidase activity and expressed as -fold increase relative to the luciferase activity in the absence of compounds. Values are mean ± S.D. (n = 4).

et al., 1998; Xu et al., 1999). Glipizide exhibits a slightly different binding mode compared with gliquidone and glimepiride, in that the sulfonyl group does not superpose to the carboxylate of farglitazar. In this case, two hydrogen bonds are formed by the urea oxygen atom with Tyr473 and with His449, whereas the deprotonated sulfonamide nitrogen forms a hydrogen bond with Ser289. Here, the deprotonated amide seems to be almost as good a mimic of farglitazar's carboxylate as the sulfonyl group is in the other examples.

Many sulfonylureas are predicted to bind PPARγ, with pK_i values ranging from 3.7 to 8.8 (Table 1). As in the case of the glinides, smaller sulfonylureas such as tolbutamide and chlorpropramide (molecular weight <300) are assigned lower binding affinities (pK_i, ~4) than larger ones such as glimepiride, glipizide, and glisamuride (molecular weight >400, pK_i, ~6–8). The larger molecules form a higher number of favorable contacts to the hydrophobic walls of the receptor's binding cavity.

Sulfonylureas and Glinides Bind to PPARγ. The predicted PPARγ ligands gliquidone, glipizide, glimepiride, repaglinide, nateglinide, and mitiglinide, as well as two known ligands, linoleic acid, an endogenous agonist, and pioglitazone, a synthetic drug used in the treatment of type 2 diabetes (all selected for commercial availability), were tested in a PPARγ competitor binding assay. Gliquidone, glimepiride, repaglinide, nateglinide, pioglitazone, and linoleic acid bind to PPARγ and completely displace the reference ligand at different concentrations. The pIC_{50} values resulting from this experiment are 5.1 for gliquidone, 3.9 for glimepiride, 2.8 for repaglinide, 3.5 for nateglinide, 6.5 for pioglitazone, and 6.6 for linoleic acid. Glipizide and mitiglinide partially displace the reference ligand at concentrations between 500 and 2000 μM (maximal concentration measured), but IC_{50} values could not be determined.

Sulfonylureas and Glinides Activate PPARγ in a Cell-Based Transactivation Assay. The eight compounds measured in the binding assay were also tested in a cell-based transactivation assay for PPARγ agonistic activity. All tested compounds activate PPARγ, albeit at various concentrations. Figure 3 reports the increase in gene expression induced by these compounds.

Among the sulfonylureas tested, gliquidone is the most potent PPARγ agonist (pEC_{50} 5.0), followed by glipizide (pEC_{50} 4.6) and glimepiride (pEC_{50} 4.0). Among the tested glinides, repaglinide shows the highest potency (pEC_{50} 4.8), followed by nateglinide (pEC_{50} 4.0) and mitiglinide (pEC_{50} 3.7). As for the standard agonists, pioglitazone (pEC_{50} 6.0) was found to be far more active than linoleic acid (pEC_{50} 3.2). For pioglitazone, PPARγ activity was reported at concentrations approximately 5 times lower than those found here (Ferry et al., 2001; Minoura et al., 2004; Inukai et al., 2005). Hence, the sensitivity of our experimental setup may be somewhat low, and the true minimum concentrations of the drugs needed for PPARγ activation may be lower than found here.

Ranking the compounds by decreasing potency, pioglitazone is followed by the sulfonylureas (gliquidone, glipizide, glimepiride), the glinides (repaglinide, nateglinide, mitiglinide), and by linoleic acid. Gliquidone approaches pioglitazone in terms of potency, reaching similar agonistic activity at a concentration 1 order of magnitude higher.

Sulfonylureas and Glinides Enhance PPARγ-Induced Target Gene Expression. The effects of gliquidone, glipizide, nateglinide, repaglinide, pioglitazone, and rosiglitazone on the expression of PPARγ target genes were measured in 3T3-L1 fibroblasts. For the sulfonylureas and glinides, concentrations bracketing EC_{50} values from the activation study were chosen (see *Materials and Methods*). Three bona fide target genes of PPARγ (Knouff and Auwerx, 2004) were selected for analysis by quantitative reverse transcription-polymerase chain reaction: adiponectin, aP2, and GLUT4. Gliquidone, nateglinide, and glipizide significantly enhanced the expression of these genes. For these three compounds, maximal induction was observed at the lowest measured concentration (10 μM for gliquidone, 50 μM for nateglinide, and 50 μM for glipizide). In contrast, repaglinide showed no induction at concentrations ranging from 50 to 200 μM. Figure 4 shows the results for the selected sulfonylureas and glinides, together with pioglitazone as positive control. The induction of gene expression is reported relative to that observed in the presence of 1 μM rosiglitazone, a strong thiazolidinedione PPARγ agonist. Gliquidone is as potent as pioglitazone and at 10 μM causes approximately 80% of the induction observed in the presence of 1 μM rosiglitazone. Nateglide and glipizide show somewhat lower activities (between 30% and 70% compared with 1 μM rosiglitazone) at higher concentrations compared with gliquidone.

Acidified Sulfonamides Other Than Sulfonylureas Are Docked to PPARγ with a High Binding Affinity. Because the docking study did not reveal any significant role in PPARγ binding for the second N atom of the sulfonylureas, we replaced this terminal NH in silico with CH_2, arriving at carbon analogs of glimepiride, glisamuride, and glibenclamide (*N*-acylsulfonamides; Fig. 5). These compounds were subjected to the docking procedure, which resulted in predicted pK_i values of 7.2, 7.2, and 6.9, respectively.

TABLE 1

Calculated pK_i values for the binding affinities of 23 sulfonylureas docked to PPARγ

Compounds were docked into the PPARγ binding site using the AutoDock software. For each ligand, the right column reports the pK_i of the best docked pose. For details on pose selection, see *Materials and Methods*.

Compound	Calculated pK_i
Glisamuride	8.8
CS 476	8.3
Glicaramide	8.1
Spc 5002	8.0
Glisindamide	7.8
Glimepiride	7.7
Gliquidone	7.5
71 5w	7.5
Glisentide	7.1
Glibenclamide	7.1
Glisoxepide	6.8
Glipizide	6.5
Glisolamide	6.4
Gliclazide	5.9
Glicondamide	5.6
Glibornuride	5.4
Acetohexamide	5.0
Metahexamide	4.8
Tolazamide	4.8
Tosifen	4.7
Tolbutamide	4.0
Carbutamide	3.7
Chlorpropamide	3.7

For C-glimepiride and C-glibenclamide pK_i values are close to those obtained for the parent sulfonylureas, whereas C-glisamuride exhibits a somewhat lower pK_i than glisamuride. In these three cases, the binding mode of the polar moiety of analogs was very similar to that of the parent sulfonylureas (i.e., to Fig. 2, D and E).

Discussion

The major results of this work are that several sulfonylurea and glinide drugs bind to and activate PPARγ in vitro and that a detailed three-dimensional binding mode underlying this activation is proposed. Experimental evidence for direct binding to PPARγ has been provided in a competitor binding assay, whereas PPARγ agonistic activity was measured both in a transactivation assay and by observing target gene levels in 3T3-L1 cells. In all these experiments, gliquidone showed the strongest PPARγ agonistic activity among the measured sulfonylureas and glinides.

While this study was underway, two sulfonylureas, glimepiride and glibenclamide, were reported to activate PPARγ (Fukuen et al., 2005; Inukai et al., 2005). Our work provides strong evidence that additional sulfonylureas, as well as glinides (which equally target the sulfonylurea receptor), can bind and activate PPARγ and allows the interpretation of binding data based on docking results.

Sulfonylureas and glinides are standard treatments for type 2 diabetes. So far, members of these classes were presumed to act by a mechanism independent of PPARγ. According to this mechanism, they bind to the sulfonylurea receptor SUR1 in pancreatic islet β cells, closing K^+ channels and leading to increased insulin production (Farret et al., 2005). In contrast, here we provide evidence that binding to and activating PPARγ may be a new mode of action for at least some of these drugs, resulting in enhanced insulin sensitivity in peripheral tissue. This discovery opens new pharmacological perspectives for drugs targeting both SUR1 and PPARγ.

For this hypothesis to be useful from a clinical point of view, it is important that the minimal drug concentrations required for PPARγ activity are reached under pharmacological treatment.

According to our measurements, gliquidone starts exhibiting a significant PPARγ agonistic activity at a concentration of 5 μM. The mean maximal plasma concentration (C_{max}) of gliquidone measured in diabetic patients treated with a 30-mg dose is 1.2 μM, with a range going from 0.2 to 4.0 μM (von Nicolai et al., 1997). The maximum recommended single dose of gliquidone is 60 mg, and the maximum daily dose is 180 mg (Anonymous, 2001). Hence, we can conclude that gliquidone activates PPARγ at pharmacologically relevant concentrations.

For glipizide, which activates PPARγ at 10 μM, measured C_{max} values are 1.0 ± 0.3 μM in patients treated with a 5-mg dose (Jaber et al., 1996). Glipizide C_{max} values are approximately 40% higher in Chinese than in white patients (Jönsson et al., 2000). The suggested maximal single dose of glipizide is 15 mg (40 mg is the maximum daily dose; http://www.pfizer.com/pfizer/download/uspi_glucotrol.pdf). This may lead to glipizide concentrations in the plasma where PPARγ activation starts being significant.

C_{max} values for glimepiride can reach 1 μM after a single 8-mg dose (Langtry and Balfour, 1998), which is the suggested maximum single dose (http://www.fda.gov/cder/foi/label/2002/20496s7lbl.pdf). This is 2 orders of magnitude below the glimepiride concentration required for PPARγ activation according to our measurements (100 μM). However, in similar experiments, other authors reported glimepiride PPARγ agonistic activity at 1 and 10 μM (Fukuen et al., 2005; Inukai et al., 2005).

For nateglinide, a C_{max} value of 18 μM has been reported in patients treated with a 120-mg dose (Luzio et al., 2001; Weaver et al., 2001; McLeod, 2004). The maximum recommended single dose of nateglinide is 180 mg (http://www.starlix.info/starlix/content/pages/basic.php). According to our measurements, nateglinide starts exhibiting PPARγ agonistic activity between 10 and 100 μM. Hence, phar-

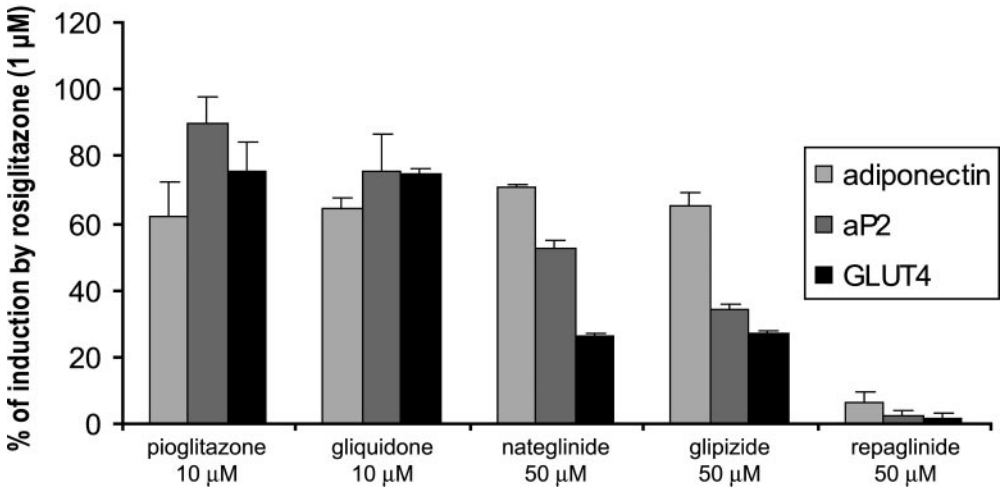

Fig. 4. Effect of selected compounds on the expression of PPARγ target genes. Mouse 3T3-L1 fibroblasts were were induced to differentiate and simultaneously treated with two common PPARγ agonists (1 μM rosiglitazone and 10 μM pioglitazone), as well as with two sulfonylureas (gliquidone and glipizide) and two glinides (repaglinide and nateglinide) at increasing concentrations (see *Materials and Methods*). Five days after induction, cells were harvested and expression levels of three PPARγ target genes (adiponectin, aP2, and GLUT4) were measured using reverse transcription-polymerase chain reaction. For each treatment, the lowest effective dose is shown (10 μM for gliquidone, 50 μM for nateglinide and repaglinide, 100 μM for glipizide). Data are normalized using cyclophilin expression and shown relative to the induction observed with 1 μM rosiglitazone. Values are mean ± S.D. (n = 3).

macological concentrations of nateglinide may be sufficient for activating PPARγ. Indeed, PPARγ activation might explain the beneficial effects on insulin resistance recently observed in patients with diabetes treated with nateglinide (Hazama et al., 2006).

Repaglinide levels in the plasma can be as high as 0.4 μM after a 4-mg dose, which is the highest recommended single dose (Owens et al., 2000; Culy and Jarvis, 2001; Hatorp, 2002). This concentration is below the minimal repaglinide concentration (around 10 μM) at which we observed PPARγ activation. Hence, repaglinide does not seem to show PPARγ activity under pharmacological treatment.

Mitiglinide starts exhibiting PPARγ agonistic activity at between 100 and 250 μM. This concentration is above the C_{max} value measured in a patient treated with an unspecified dose of mitiglinide (1.6 μM) (Anonymous, 2004).

To summarize, there is evidence that gliquidone, glipizide, and nateglinide may activate PPARγ at pharmacologically relevant concentrations, whereas glimepiride, repaglinide, and mitiglinide only activate PPARγ at concentrations higher than the those reached under clinical circumstances.

Our computational results strongly suggest to experimentally test members of the third compound class considered, *N*-acylsulfonamides (*N*-sulfonylcarboxamides), such as C-glimepiride, C-glisamuride, and C-glibenclamide, for PPARγ activity. These compounds are not yet synthesized. One recent publication, however, described a *N*-acylsulfonamide, FK614, as both an insulin sensitizer and PPARγ activator (Minoura et al., 2004). When subjected to our docking procedure, FK614 was assigned a pK_i value of 6.4.

As illustrated in Fig. 6, common properties of carboxylic acids (**1**) and thiazolidinediones (**2**), the major PPARγ ligand classes at present, are their acidity and the hydrogen bond acceptor potential of their deprotonated forms. According to X-ray analyses of PPARγ-ligand complexes, these properties are highly important for binding (Nolte et al., 1998; Gampe et al., 2000; Cronet et al., 2001; Xu et al., 2001; Sauerberg et al., 2002; Ebdrup et al., 2003). Sulfonamides (**3**) are not sufficiently acidic for binding unless acidified by a substituent such as an acyl group −C(=O)R′ that stabilizes the conjugate base by further delocalizing the negative charge. At the same time, such a substituent provides another H bond acceptor atom, the carbonyl oxygen. The compound classes shown or predicted here to be PPARγ ligands, sulfonylureas [**4,** R′ = (substituted) amino] and *N*-acylsulfonamides [**4,** R′ = (substituted) alkyl or aryl, pK_a values of ~5] smoothly fit into this picture. Other compound classes exhibiting similar or higher acidity and similar H acceptor ability of their anions were recently shown to be PPARγ ligands [oxazolidinediones (Momose et al., 2002b), tetrazoles (Momose et al., 2002a), phosphates, and thiophosphates (Durgam et al., 2005)]. Circumstantial evidence for our views is provided by the observation that the succinimide analog of an antihyperglycemic thiazolidinedione (**2**, but S replaced by CH_2, pK_a 9.7), as well as the corresponding *N*-methylthiazolidinedione, are both inactive (Cantello et al., 1994). Thus, a unified understanding of the PPARγ binding ability of seemingly disparate compound classes is emerging.

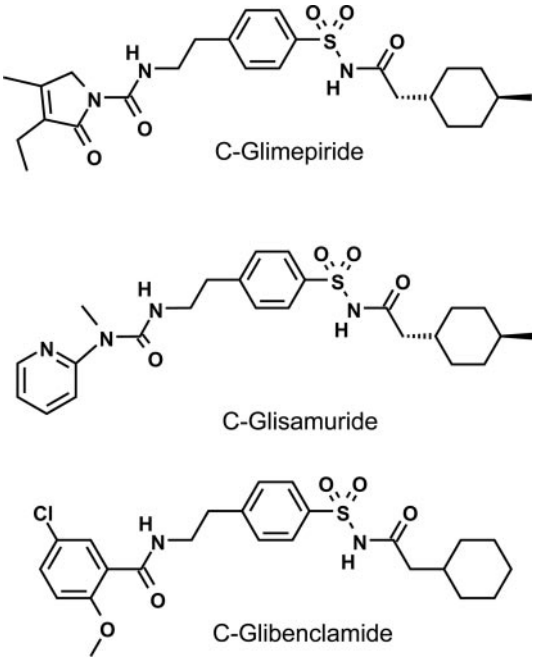

Fig. 5. Structures of carbon analogs of glimepiride, glisamuride, and glibenclamide (*N*-acylsulfonamides).

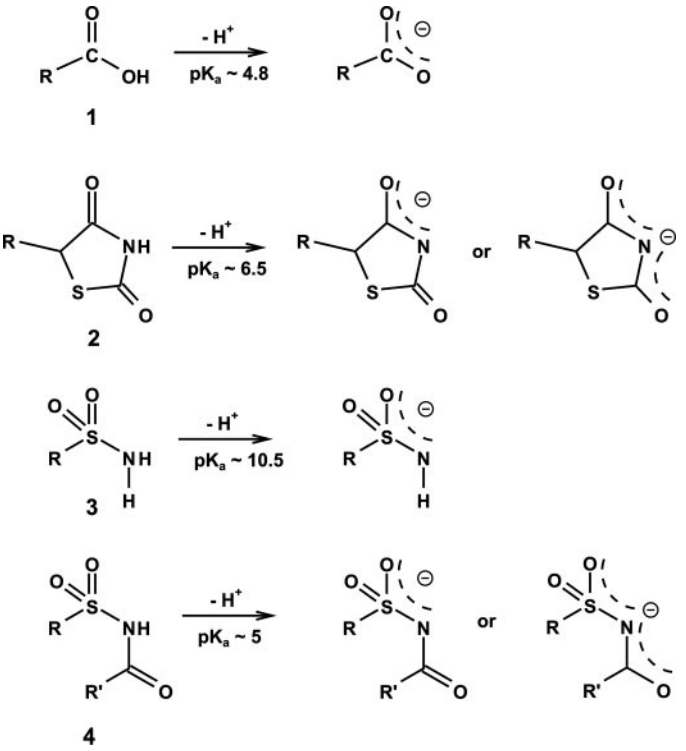

Fig. 6. Acid strength and hydrogen bond acceptor ability of some PPARγ agonist compound classes. Analogy in acid strength and in hydrogen bond acceptor ability between carboxylic acids (**1**), thiazolidinediones (**2**), sulfonamides (**3**), sulfonylureas [(**4**), R′ = (substituted) amino], and *N*-acylsulfonamides [(**4**), R′ = (substituted) alkyl or aryl], or the respective anions. In the anions, oxygen and nitrogen atoms participating in charge delocalization as indicated are potential hydrogen bond acceptors. Both sulfonyl oxygen atoms are equivalent. For simplicity, charge delocalization onto the second sulfonyl oxygen atom is not shown.

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