

## **Sulfonylureas and Glinides Exhibit PPAR $\gamma$ Activity: A Combined Virtual Screening and Biological Assay Approach\***

**Marco Scarsi<sup>†</sup>, Michael Podvinec<sup>†</sup>, Adrian Roth, Hubert Hug, Sander Kersten,  
Hugo Albrecht, Torsten Schwede, Urs A. Meyer and Christoph Rücker**

*Biozentrum, University of Basel, Klingelbergstr. 50-70,  
CH-4056 Basel, Switzerland (M.S., M.P., A.R., T.S., U.A.M., C.R.)*

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*Swiss Institute of Bioinformatics, Klingelbergstr. 50-70,  
CH-4056 Basel, Switzerland (M.S., M.P., T.S.)*

-

*Hoffmann-La Roche AG, CH-4070 Basel (A.R.)*

-

*TheraStrat AG, Gewerbestr. 25, CH-4123 Allschwil, Switzerland (H.H.)*

-

*DSM Nutritional Products, Human Nutrition and Health, Wurmisweg 576,  
CH-4303 Kaiseraugst, Switzerland (H.H.)*

-

*Nutrition, Metabolism and Genomics Group, Wageningen University, PO BOX 8129  
6700 EV Wageningen, the Netherlands (S.K.)*

-

*BioFocus DPI, Gewerbestr. 16, CH-4123 Allschwil, Switzerland (H.A.)*

<sup>†</sup>*M.S. and M.P. contributed equally to this work.*

## Running Title:

# Sulfonylureas and Glinides Exhibit PPAR $\gamma$ Activity

Address correspondence to: Marco Scarsi, Biozentrum, University of Basel, Klingelbergstr. 50-70,

CH-4056 Basel, Switzerland, Tel: +41-61-267-1469; Fax: +41-61-267-1584; E-Mail:

marco.scarsi@unibas.ch

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**Non-standard abbreviations:** PPAR, peroxisome proliferator-activated receptor; SUR1, sulfonylurea receptor 1; LBD, ligand binding domain; IC<sub>50</sub>, concentration at which 50% of the reference ligand is displaced; pIC<sub>50</sub>, negative decadic logarithm of IC<sub>50</sub>; DMEM, Dulbecco's Modified Eagle's Medium; FBS; DMEM/F12, Dulbecco's Modified Eagle's Medium/Ham's F-12; DMSO, Dimethylsulfoxide; PPRE, PPAR response element; EC<sub>50</sub>, concentration at which 50% of the maximal gene expression is induced; pEC<sub>50</sub>, negative decadic logarithm of EC<sub>50</sub>; C<sub>max</sub>, mean maximal plasma concentration; FK614, 3-(2,4-dichlorobenzyl)-2-methyl-N-(pentylsulfonyl)-3H-benzimidazole-5-carboxamide; aP2, adipocyte fatty acid-binding protein gene; GLUT4, glucose transporter-4 gene.

## Abstract

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

## Introduction

Poor eating habits and sedentary lifestyle have lead to an increasing prevalence of metabolic disorders, such as type 2 diabetes mellitus, which is attaining epidemic proportions (WHO, 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 often is 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 co-activators and co-repressors, 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 PPAR $\alpha$ , PPAR $\gamma$ , and PPAR $\delta$ , that are coded by different genes, exhibit tissue-specific expression patterns and are associated with various functions. Of these, PPAR $\gamma$  is mostly expressed in adipose tissue, where it is essential in adipocyte differentiation and controls the storage of fatty acids, increasing triglyceride synthesis and storage within adipocytes. Additionally, there is strong evidence that PPAR $\gamma$  regulates glucose homeostasis (Rangwala and Lazar, 2004; Wang and Tafuri, 2003; Willson et al., 2000; Willson et al., 2001). Activation of PPAR $\gamma$  improves insulin resistance, and therefore PPAR $\gamma$  is an established molecular target for the treatment of type 2 diabetes (Perfetti and D'Amico, 2005; Staels and Fruchart, 2005).

For PPAR $\gamma$ , several unsaturated fatty acids have been proposed as natural ligands, in particular prostaglandins such as 15-deoxy- $\Delta^{12,14}$ -PGJ<sub>2</sub> (Ferry et al., 2001), nitrolinoleic acids such as 10-nitrolinoleic acid (Schopfer et al., 2005), and putative metabolites of docosahexaenoic acid such as 4-OH DHA (Yamamoto et al., 2005). A few synthetic PPAR $\gamma$  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 Figure 1 for chemical structures. All PPAR $\gamma$  agonists in clinical use or development, and in fact most known PPAR $\gamma$  agonists are either thiazolidinediones or carboxylic acids (Martin et al., 2005). Many drug therapies targeting PPAR $\gamma$  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 PPAR $\gamma$  ligand and drug candidate, farglitazar (see Figure 1), had to be discontinued in phase III for the emergence of edema (Parker, 2002; Shi et al., 2005). Therefore demand is increasing for new PPAR $\gamma$  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 PPAR $\gamma$  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 PPAR $\gamma$  ligands, and prompted us to concentrate on these two drug classes, screening several more members thereof. Most of these compounds showed good affinities to PPAR $\gamma$  *in silico*. In a second step, we found that selected sulfonylureas and glinides bind to PPAR $\gamma$  and enhance PPAR $\gamma$ -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 (that is *N*-acylsulfonamides), resulting in the prediction that they would bind PPAR $\gamma$  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  $\beta$ -cells, triggering the closure of the nearby potassium channel, which in turns leads the  $\beta$ -cell to increase insulin secretion (Farret et al., 2005). Our discovery that

some insulin secretagogue drugs activate PPAR $\gamma$  has attractive implications for the pharmacological treatment of type 2 diabetes. Moreover, sulfonylureas and *N*-acylsulfonamides are new classes of PPAR $\gamma$  agonists.

## Materials and Methods

**Virtual screening database:** The TheraStrat AG inhouse database (Gut and Bagatto, 2005; Hug et al., 2003), contains most marketed drugs and many of their metabolites (approximately 8000 compounds). The freely available ChEMBL database contains about 6000 bioactive compounds (ChEMBL, 2004).

**Ligand docking:** Each compound was docked into the PPAR $\gamma$  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<sub>i</sub> value is calculated. The PPAR $\gamma$  3D structure was obtained from PDB entry 1FM9. This is a 2.1 Å resolution crystal structure of the heterodimer of the human RXR $\alpha$  and PPAR $\gamma$  ligand binding domains, bound to 9*cis*-retinoic acid and farglitazar, respectively, together with coactivator peptides (Gampe et al., 2000). The PPAR $\gamma$ -farglitazar complex was imported in MOE (MOE, 2006) 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, 2006). For method verification and calibration we docked to PPAR $\gamma$  a set of 121 carboxylic acids that are PPAR $\gamma$  agonists with known experimental binding affinities, a collection detailed in our earlier work (Rucker 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<sub>i</sub> 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). Since 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 intercept. 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. These constraints are justified by the facts that in all PPAR $\gamma$ -ligand complexes with known X-ray structures such hydrogen bonds seem to be essential for binding (Cronet et al., 2001; Gampe et al., 2000; Nolte et al., 1998; Sauerberg et al., 2002; Xu et al., 2001), and that the hydrogen bond to Tyr473 was proposed to play a vital role for PPAR $\gamma$  co-activator recruitment (Cronet et al., 2001).

**Reagents and plasmids.** Standard cell culture and transfection reagents were purchased from Invitrogen (Carlsbad, USA). 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 from Sigma-Aldrich, nateglinide from Toronto Research Chemicals Inc. (North York, Canada), mitiglinide and pioglitazone from Molekula Ltd. (Dorset, UK). The 3xPPRE-Luc vector was kindly provided by Ron Evans (The Salk Institute for Biological Studies, San Diego, USA). The expression vector encoding human PPAR $\gamma$  was provided by Walter Wahli (Centre Intégratif De Genomique, Lausanne, Switzerland).

**Ligand binding assay.** The Green PolarScreen PPAR Competitor Assay (Invitrogen, Carlsbad, USA) was used according to the manufacturer's instructions. This cell-free assay is based on purified recombinant human PPAR $\gamma$  ligand-binding domain (LBD) and a selective fluorescent PPAR $\gamma$  ligand. The LBD-ligand complex exhibits high fluorescence polarization, which is lost upon ligand displacement by non-labelled competitors. Millipolarization ( $mP$ ) values for different competitors were determined in a ViewLux reader 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 is the median  $mP$  value 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 (4500mg/l glucose), supplemented with 10% FBS and 50 U Penicillin/Streptomycin. Three days before transfection, cells were sterol-depleted by exchanging the culture medium to DMEM/F12 without Phenol Red, supplemented with 10% charcoal-stripped FBS and 50 U Penicillin/Streptomycin. Cells were plated in a 96-well dish with a density of 500'000 cells/ml (100  $\mu$ l per well). DNA transfection was carried out in OptiMem I without Phenol Red using Lipofectamine. Each well received 8ng expression vector, 20ng reporter vector and 60ng  $\beta$ -galactosidase vector. 24h after transfection drugs dissolved in DMSO were added in DMEM/F12 without Phenol Red, supplemented with 10% charcoal-stripped delipidated FBS (Sigma-Aldrich, Buchs, Switzerland), and 50 U Penicillin/Streptomycin. 16h after addition of drugs, cells were lysed in CAT lysis buffer (Promega, Catalys AG, Wallisellen, Switzerland). Supernatants were analyzed for luciferase activity by addition of luciferase reagent (Promega, Catalys AG). Background normalization was carried out by measuring  $\beta$ -galactosidase activity as previously described (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 PPAR $\gamma$  target gene expression induced at different concentrations. A four-parametric sigmoidal curve was fitted to each data set using Prism® software from GraphPad™ (San Diego, USA), and the  $EC_{50}$  value was determined from the fitted curve. Experiments were performed in quadruplicate, and error bars represent standard deviations.

**Measurement of PPAR $\gamma$  target gene expression.** 3T3-L1 fibroblasts were purchased from European Collections of Cell Cultures. They were amplified in DMEM/10% calf serum and subsequently seeded into 6-well plates. Two days after the cells reached confluency, the medium was changed to DMEM/10% fetal calf serum containing 0.5 M isobutylmethylxanthine, 2  $\mu$ g/mL insulin (Actrapid,



Novo Nordisk), and 0.5  $\mu$ M 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  $\mu$ g/mL insulin, to which the experimental compounds were freshly added. Compounds were tested at the following concentrations: rosiglitazone (1  $\mu$ M), pioglitazone (10  $\mu$ M), gliquidone (10  $\mu$ M), glipizide (100  $\mu$ M, 200  $\mu$ M), nateglinide (50  $\mu$ M, 200  $\mu$ M), repaglinide (50  $\mu$ M, 100  $\mu$ M, 200  $\mu$ M). The cells were harvested in Trizol (Invitrogen, Breda, the Netherlands) 5 days after induction, and RNA was isolated by the standard procedure. 1  $\mu$ g of RNA was used for cDNA synthesis using iScript (Biorad, 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; aP2-reverse: AATCCCCATTTACGCTGATG; GLUT4-forward: GGAAGGAAAAGGGCTATGCTG; GLUT4-reverse: TGAGGAACCGTCCAAGAATGA; Cyclophilin-forward: TGTCTTTGGAACCTTGTCTGCAA; Cyclophilin-reverse: CAGACGCCACTGTCGCTTT; Adiponectin-forward: GCAGAGATGGCACTCCTGGA; Adiponectin-reverse: CCCTTCAGCTCCTGTCATTCC.

## Results

In a first step, all compounds in the TheraStrat and ChEMBL databases were docked to PPAR $\gamma$ . 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 PPAR $\gamma$  with a high binding affinity.**

Figures 2A, 2B, and 2C depict repaglinide, nateglinide and mitiglinide, respectively, docked into PPAR $\gamma$  and superimposed to the farglitazar complex X-ray structure (for structures see Figure 3). The predicted bound conformations to PPAR $\gamma$  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 PPAR $\gamma$ , as farglitazar does. Repaglinide forms several hydrophobic contacts in the large apolar cavity (bottom left of Figure 2A) that in the case of farglitazar is occupied by its long hydrophobic tail. Nateglinide and mitiglinide fit well the smaller hydrophobic cavity (bottom right of Figure 2B and 2C) that in the farglitazar complex is occupied by the benzoylphenyl group.

Repaglinide, mitiglinide, nateglinide, and meglitinide are predicted to bind PPAR $\gamma$  with pK<sub>i</sub> values of 7.2, 6.3, 5.9, and 5.0, respectively. The smaller molecules mitiglinide, nateglinide, and meglitinide (molecular weight < 350) bind at a lower affinity compared to the larger repaglinide (molecular weight 453), as the latter forms more favorable contacts between the hydrophobic cavities of the PPAR $\gamma$  binding site and its large hydrophobic moieties.

Figures 2D, 2E, and 2F show gliquidone, glimepiride, and glipizide, respectively, docked into PPAR $\gamma$  and superimposed to the farglitazar complex X-ray structure (for structures see Figure 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. Notably, 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, since this part of the binding cavity is wide, allowing some conformational freedom for the ligand (Nolte et al., 1998; Xu et al., 1999). Glipizide exhibits a slightly different binding mode compared to 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, while 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 is the sulfonyl group in the other examples.

Many sulfonylureas are predicted to bind PPAR $\gamma$  with pK<sub>i</sub> 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<sub>i</sub> ~4) than larger ones such as glimepiride, glipizide, and glisamuride (molecular weight > 400, pK<sub>i</sub> ~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 PPAR $\gamma$**

The predicted PPAR $\gamma$  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 PPAR $\gamma$  competitor binding assay. Gliquidone, glimepiride, repaglinide, nateglinide, pioglitazone and linoleic acid bind to PPAR $\gamma$  and completely displace the reference ligand at different concentrations. The pIC<sub>50</sub> 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  $\mu$ M (maximal concentration measured), but IC<sub>50</sub> values could not be determined.

### **Sulfonylureas and glinides activate PPAR $\gamma$ in a cell-based transactivation assay.**

The eight compounds measured in the binding assay were also tested in a cell-based transactivation assay for PPAR $\gamma$  agonistic activity. All tested compounds activate PPAR $\gamma$ , 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 PPAR $\gamma$  agonist (pEC<sub>50</sub> 5.0), followed by glipizide (pEC<sub>50</sub> 4.6) and glimepiride (pEC<sub>50</sub> 4.0). Among the tested glinides, repaglinide shows

the highest potency (pEC<sub>50</sub> 4.8), followed by nateglinide (pEC<sub>50</sub> 4.0) and mitiglinide (pEC<sub>50</sub> 3.7). As to the standard agonists, pioglitazone (pEC<sub>50</sub> 6.0) was found far more active than linoleic acid (pEC<sub>50</sub> 3.2). For pioglitazone, PPAR $\gamma$  activity was reported at concentrations about 5 times lower than found here (Ferry et al., 2001; Inukai et al., 2005; Minoura et al., 2004). Hence, the sensitivity of our experimental setup may be somewhat low, and the true minimum concentrations of the drugs needed for PPAR $\gamma$  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 one order of magnitude higher.

#### **Sulfonylureas and glinides enhance PPAR $\gamma$ -induced target gene expression.**

The effects of gliquidone, glipizide, nateglinide, mitiglinide, pioglitazone, and rosiglitazone on the expression of PPAR $\gamma$  target genes were measured in 3T3-L1 fibroblasts. For the sulfonylureas and glinides, concentrations bracketing EC<sub>50</sub> values from the activation study were chosen (see materials and methods). Three bona fide target genes of PPAR $\gamma$  (Knouff and Auwerx, 2004) were selected for analysis by quantitative RT-PCR: 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  $\mu$ M for gliquidone, 50  $\mu$ M for nateglinide, and 100  $\mu$ M for glipizide). In contrast, repaglinide did not show any induction at concentrations ranging from 50  $\mu$ M to 200  $\mu$ M. 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  $\mu$ M of rosiglitazone, a strong thiazolidinedione PPAR $\gamma$  agonist. Gliquidone is as potent as pioglitazone and at 10  $\mu$ M causes about 80% of the induction observed in the presence of 1  $\mu$ M of rosiglitazone. Nateglinide and glipizide show somewhat lower activities (between 30% and 70% compared to 1  $\mu$ M of rosiglitazone) at higher concentrations compared to gliquidone.

**Acidified sulfonamides other than sulfonylureas are docked to PPAR $\gamma$  with a high binding affinity.**

Since the docking study did not reveal any significant role in PPAR $\gamma$  binding for the second N atom of the sulfonylureas, we *in silico* replaced this terminal NH by CH<sub>2</sub>, arriving at carbon analogs of glimepiride, glisamuride, and glibenclamide (N-acylsulfonamides, Figure 5). These compounds were subjected to the docking procedure, which resulted in predicted pK<sub>i</sub> values of 7.2, 7.2, and 6.9, respectively.

For C-glimepiride and C-glibenclamide pK<sub>i</sub> values are close to those obtained for the parent sulfonylureas, while C-glismuride exhibits a somewhat lower pK<sub>i</sub> than glismuride. 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 Figures 2D and 2E.

## Discussion

The major results of this work are that several sulfonylurea and glinide drugs bind to and activate PPAR $\gamma$  *in vitro*, and that a detailed 3D binding mode underlying this activation is proposed. Experimental evidence for direct binding to PPAR $\gamma$  has been provided in a competitor binding assay, while PPAR $\gamma$  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 PPAR $\gamma$  agonistic activity among the measured sulfonylureas and glinides.

While this study was underway, two sulfonylureas, glimepiride and glibenclamide, were reported to activate PPAR $\gamma$  (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 PPAR $\gamma$ , and allows the interpretation of binding data on the basis of 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 PPAR $\gamma$ . According to this mechanism, they bind to the sulfonylurea receptor SUR1 in pancreatic islet  $\beta$  cells, closing K<sup>+</sup> channels, and

leading to increased insulin production (Farret et al., 2005). In contrast, here we provide evidence that binding to and activating PPAR $\gamma$  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 PPAR $\gamma$ .

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

According to our measurements gliquidone starts exhibiting a significant PPAR $\gamma$  agonistic activity at a concentration of 5  $\mu$ M. The mean maximal plasma concentration ( $C_{\max}$ ) of gliquidone measured in diabetic patients treated with a 30 mg dose is 1.2  $\mu$ M, with a range going from 0.2 to 4.0  $\mu$ M (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 PPAR $\gamma$  at pharmacologically relevant concentrations.

For glipizide, which activates PPAR $\gamma$  at 10  $\mu$ M, measured  $C_{\max}$  values are  $1.0 \pm 0.3$   $\mu$ M in patients treated with a 5 mg dose (Jaber et al., 1996). Glipizide  $C_{\max}$  values are about 40% higher in Chinese than in Caucasian patients (Jönsson et al., 2000). The suggested maximal single dose of glipizide is 15 mg (40 mg is the maximum daily dose) (Pfizer, 2000). This may lead to glipizide concentrations in the plasma where PPAR $\gamma$  activation starts being significant.

$C_{\max}$  values for glimepiride can reach 1  $\mu$ M following a 8 mg single dose (Langtry and Balfour, 1998), which is the suggested maximum single dose (Aventis, 2001). This is two orders of magnitude below the glimepiride concentration required for PPAR $\gamma$  activation according to our measurements (100  $\mu$ M). However, from similar experiments other authors reported glimepiride PPAR $\gamma$  agonistic activity at 1  $\mu$ M and 10  $\mu$ M (Fukuen et al., 2005; Inukai et al., 2005).

For nateglinide, a  $C_{\max}$  value of 18  $\mu$ M has been reported in patients treated with a 120 mg dose (Luzio et al., 2001; McLeod, 2004; Weaver et al., 2001). The maximum recommended single dose of nateglinide is 180 mg (Novartis, 2005). According to our measurements nateglinide starts exhibiting PPAR $\gamma$  agonistic activity between 10 and 100  $\mu$ M. Hence, pharmacological concentrations of nateglinide may be sufficient for activating PPAR $\gamma$ . Indeed, PPAR $\gamma$  activation might explain the

beneficial effects on insulin resistance recently observed in diabetic patients treated with nateglinide (Hazama et al., 2006).

Repaglinide levels in the plasma can be as high as 0.4  $\mu\text{M}$  following a 4 mg dose, which is the highest recommended single dose (Culy and Jarvis, 2001; Hatorp, 2002; Owens et al., 2000). This concentration is below the minimal repaglinide concentration (around 10  $\mu\text{M}$ ) at which we observed PPAR $\gamma$  activation. Hence, repaglinide does not seem to show PPAR $\gamma$  activity under pharmacological treatment.

Mitiglinide starts exhibiting PPAR $\gamma$  agonistic activity between between 100 and 250  $\mu\text{M}$ . This concentration is above the  $C_{\text{max}}$  value measured in a patient treated with an unspecified dose of mitiglinide (1.6  $\mu\text{M}$ ) (Anonymous, 2004).

To summarize, there is evidence that gliquidone, glipizide, and nateglinide may activate PPAR $\gamma$  at pharmacologically relevant concentrations, while glimepiride, repaglinide, and mitiglinide only activate PPAR $\gamma$  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 PPAR $\gamma$  activity. These compounds are not yet synthesized. There has, however, been a recent publication describing one such *N*-acylsulfonamide, FK614, as both an insulin sensitizer and PPAR $\gamma$  activator (Minoura et al., 2004). When subjected to our docking procedure, FK614 was assigned a  $\text{pK}_i$  value of 6.4.

Common properties of carboxylic acids (**1**) and thiazolidinediones (**2**), the major PPAR $\gamma$  ligand classes at present, are their acidity and the hydrogen bond acceptor potential of their deprotonated forms, as illustrated in Figure 6. According to X-ray analyses of PPAR $\gamma$ -ligand complexes these properties are highly important for binding (Cronet et al., 2001; Ebdrup et al., 2003; Gampe et al., 2000; Nolte et al., 1998; Sauerberg et al., 2002; Xu et al., 2001). Sulfonamides (**3**) are not sufficiently acidic for binding unless acidified by a substituent such as an acyl group  $-\text{C}(=\text{O})\text{R}'$  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 PPAR $\gamma$  ligands, sulfonylureas [**4**, R' = (substituted) amino] and *N*-acylsulfonamides [**4**, R' = (substituted) alkyl or aryl, pK<sub>a</sub> 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 PPAR $\gamma$  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<sub>2</sub>, pK<sub>a</sub> 9.7), as well as the corresponding *N*-methylthiazolidinedione, are both inactive (Cantello et al., 1994). Thus, a unified understanding of the PPAR $\gamma$  binding ability of seemingly disparate compound classes is emerging.

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## Footnotes

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Person to receive reprint requests: Marco Scarsi, Biozentrum, University of Basel, Klingelbergstr. 50-70, CH-4056 Basel, Switzerland, Tel: +41-61-267-1583; Fax: +41-61-267-1584; E-Mail: marco.scarsi@unibas.ch

## Figure Legends

**Figure 1:** Structures of some endogenous and synthetic PPAR $\gamma$  agonists.

**Figure 2.** 3D structures of three glinides and three sulfonylureas docked to PPAR $\gamma$ . Repaglinide (A), nateglinide (B), mitiglinide (C), gliquidone (D), glimepiride (E), and glipizide (F) (grey ball and stick models) docked to PPAR $\gamma$  and superimposed to the farglitazar complex X-ray structure (green stick model). Of PPAR $\gamma$ , only the side chains of His323, His449, Ser289, and Tyr473 are shown (grey stick models). Compounds were docked into the PPAR $\gamma$  binding site using the AutoDock software. For each ligand the figure reports the best docked pose. For details on pose selection see “Experimental Procedures”.

**Figure 3.** Induction of PPAR $\gamma$ -mediated gene expression. The effects of three sulfonylureas (gliquidone, glipizide, and glimepiride, upper graph), three glinides (repaglinide, nateglinide, and mitiglinide, middle graph), and two standard agonists (pioglitazone and linoleic acid, bottom graph) on PPAR $\gamma$ -dependent transactivation were assayed in CV-1 cells. Cells were transfected with expression vector encoding human PPAR $\gamma$ , 3xPPRE-Luc reporter vector, and  $\beta$ -galactosidase vector as described under “Experimental Procedures”. Following 24 hours of transfection, cells were treated for 16 hours with indicated concentrations of each compound. Luciferase activity was normalized by  $\beta$ -galactosidase activity and expressed as fold increase relative to the luciferase activity in the absence of compounds. Values are mean  $\pm$  S.D. ( $n=4$ ).

**Figure 4:** Effect of selected compounds on the expression of PPAR $\gamma$  target genes. Mouse 3T3-L1 fibroblasts were induced to differentiate and simultaneously treated with two common PPAR $\gamma$  agonists (rosiglitazone 1 $\mu$ M, pioglitazone 10 $\mu$ M), as well as with two sulfonylureas (gliquidone and glipizide) and two glinides (repaglinide and nateglinide) at increasing concentrations (see methods). Five days after induction, cells were harvested and expression levels of three PPAR $\gamma$

target genes (adiponectin, aP2 and GLUT4) were measured using RT-PCR. For each treatment, the lowest effective dose is shown (10  $\mu$ M for gliquidone, 50  $\mu$ M for nateglinide and repaglinide, 100  $\mu$ M for glipizide). Data are normalized using cyclophilin expression and shown relative to the induction observed with 1  $\mu$ M rosiglitazone. Values are mean  $\pm$  S.D. (n=3).

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

**Figure 6:** Acid strength and hydrogen bond acceptor ability of some PPAR $\gamma$  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, O and N 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.

**Table 1**

**Calculated pK<sub>i</sub> values for the binding affinities of 23 sulfonylureas docked to PPAR<sub>γ</sub>.**

Compounds were docked into the PPAR<sub>γ</sub> binding site using the AutoDock software. For each ligand the right column reports the pK<sub>i</sub> of the best docked pose. For details on pose selection see “Experimental Procedures”.

Compound	Calculated pK <sub>i</sub>
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

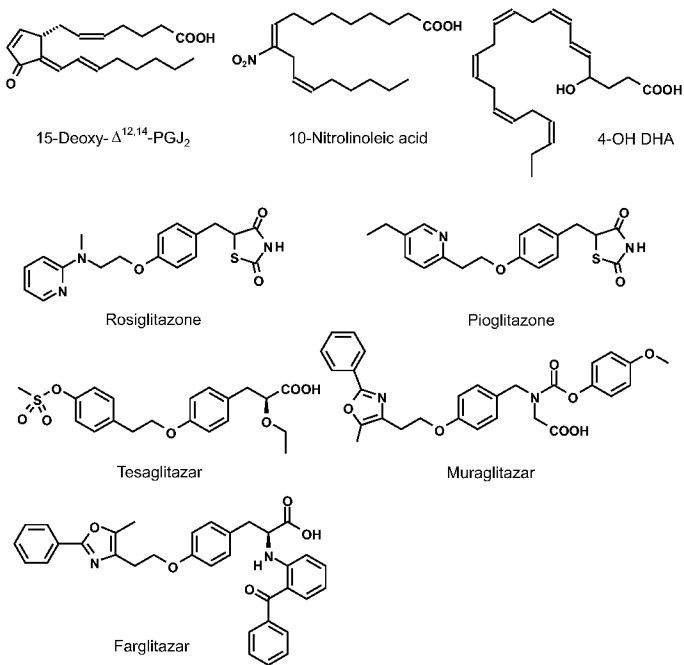


Fig.1, Scarsi *et al.*

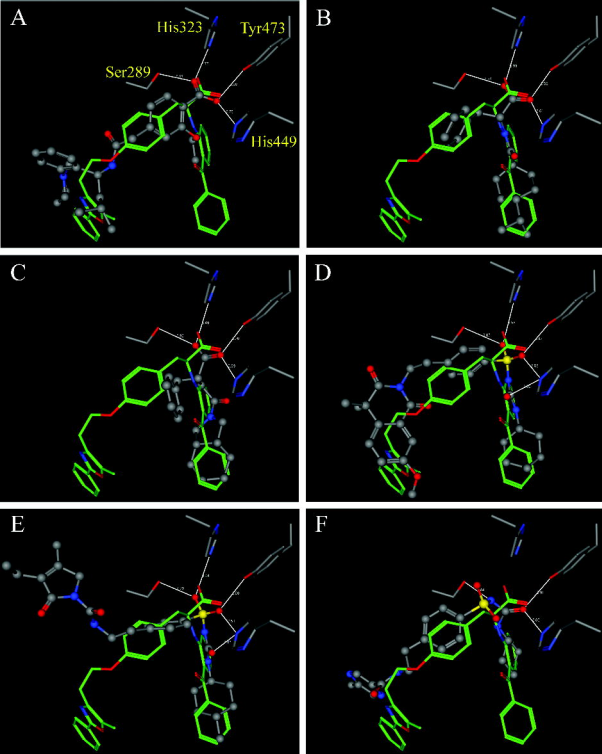


Fig.2, Scarsi *et al.*

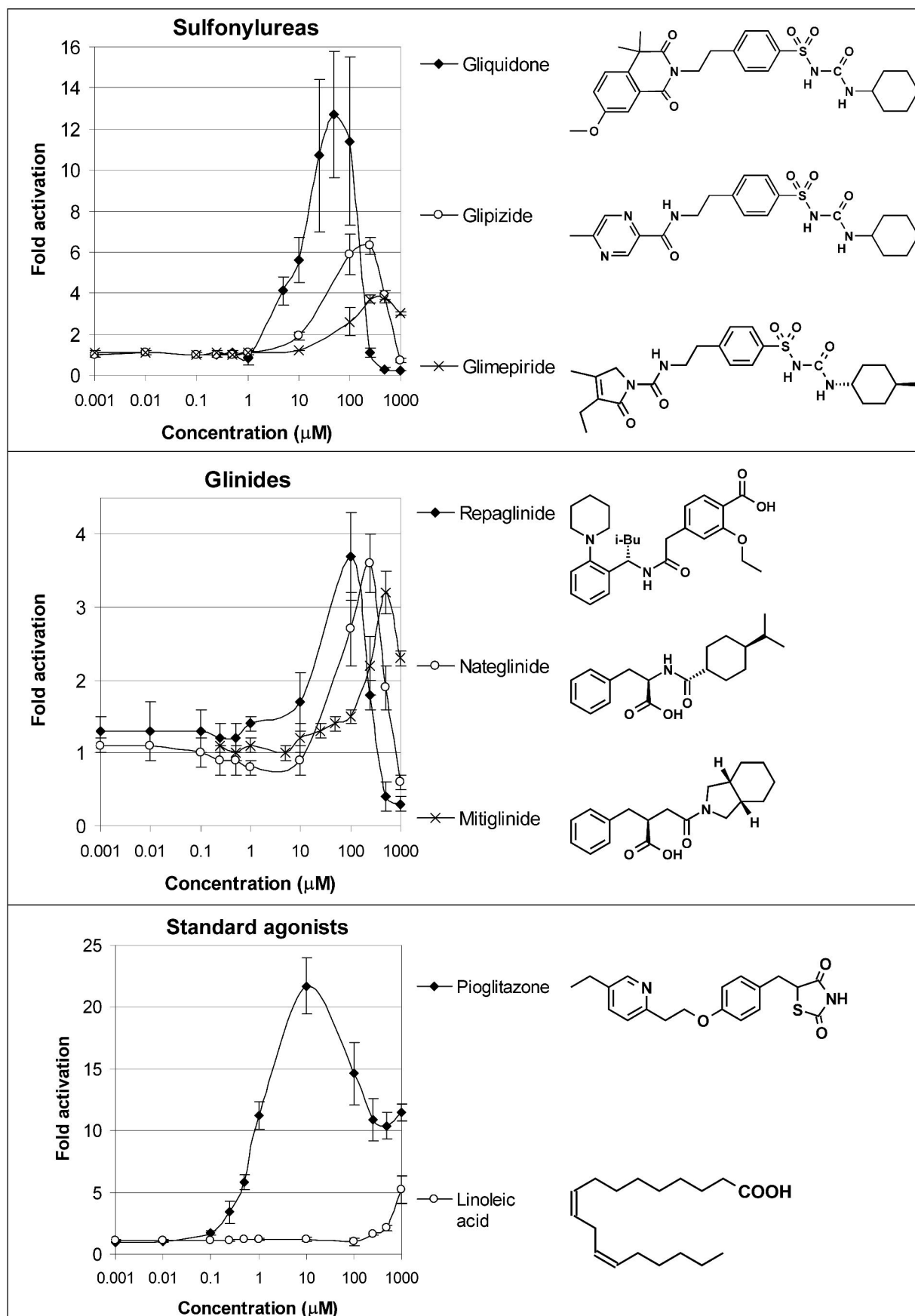


Fig. 3, Scarsi *et al.*

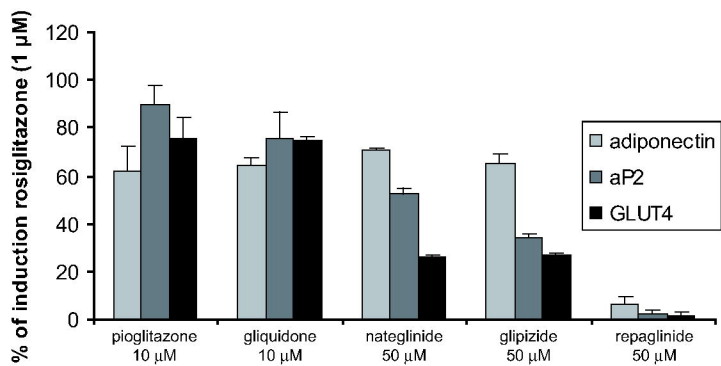


Fig. 4, Scarsi *et al.*

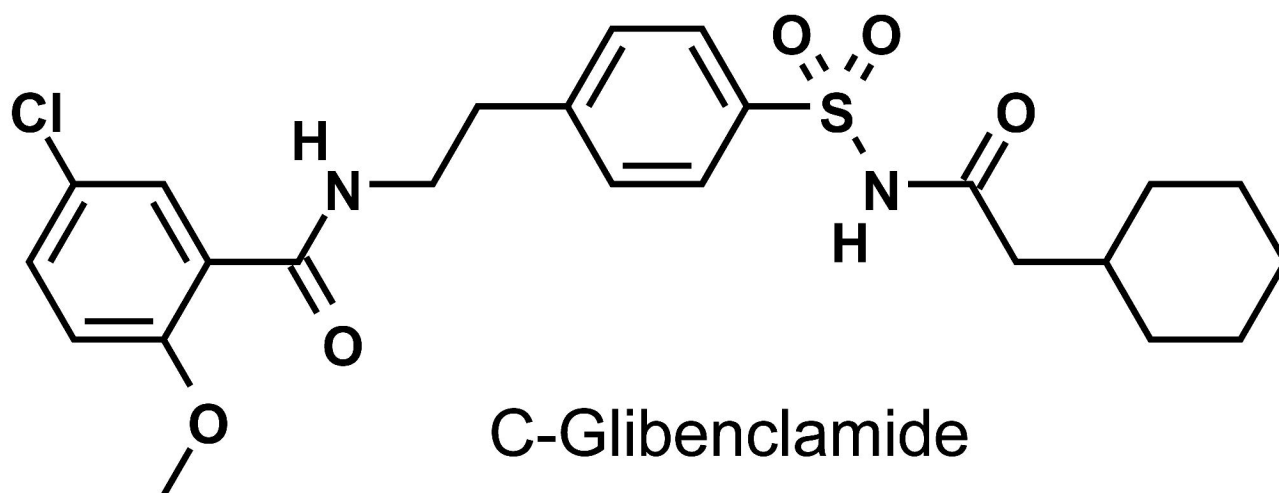
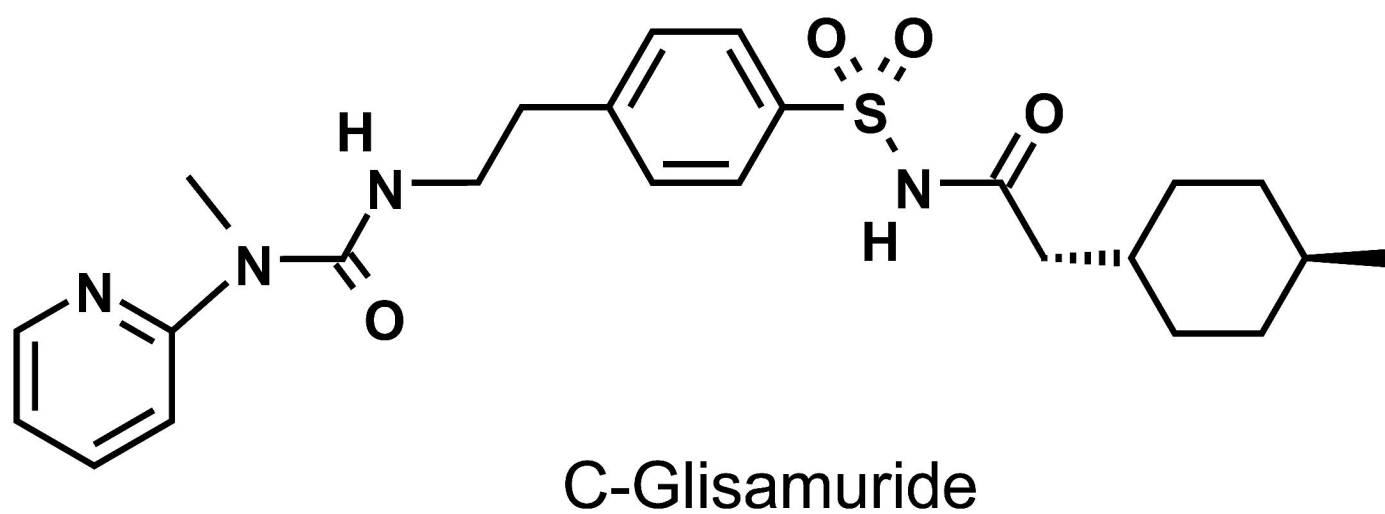
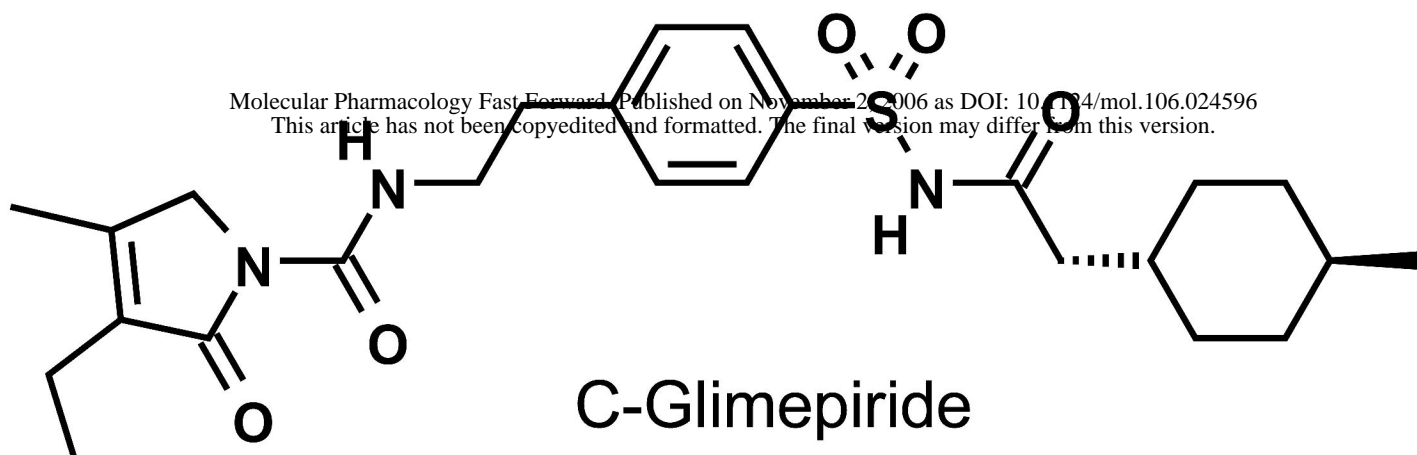


Fig. 5, Scarsi *et al.*

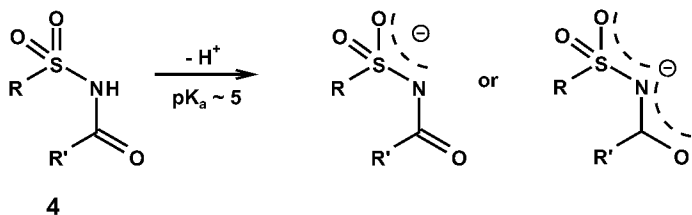
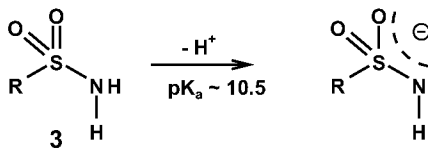
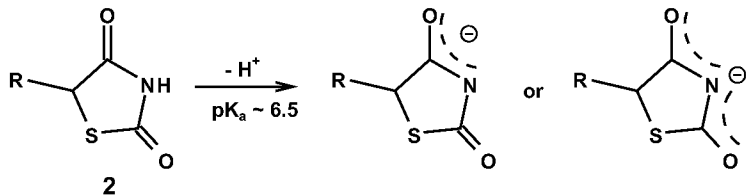
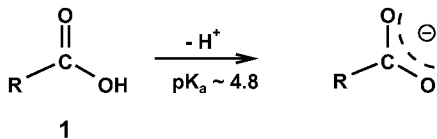


Fig. 6, Scarsi *et al.*