Pharmacophore-driven identification of PPARγ agonists from natural sources

Rasmus K. Petersen · Kathrine B. Christensen · Andreana N. Assimopoulou · Xavier Fretté · Vassilios P. Papageorgiou · Karsten Kristiansen · Irene Kouskoumvekaki

Abstract In a search for more effective and safe anti-diabetic compounds, we developed a pharmacophore model based on partial agonists of PPARγ. The model was used for the virtual screening of the Chinese Natural Product Database (CNPD), a library of plant-derived natural products primarily used in folk medicine. From the resulting hits, we selected methyl oleanonate, a compound found, among others, in *Pistacia lentiscus* var. Chia oleoresin (Chios mastic gum). The acid of methyl oleanonate, oleanonic acid, was identified as a PPARγ agonist through bioassay-guided chromatographic fractionations of Chios mastic gum fractions, whereas some other sub-fractions exhibited also biological activity towards PPARγ. The results from the present work are two-fold: on the one hand we demonstrate that the pharmacophore model we developed is able to select novel ligand scaffolds that act as PPARγ agonists; while at the same time it manifests that natural products are highly relevant for use in virtual screening-based drug discovery.

Keywords Virtual screening · PPARγ agonist · Natural compounds · Pharmacophore model

R. K. Petersen · K. Kristiansen
Department of Biology, University of Copenhagen,
Ole Maaløes Vej 5, 2200 Copenhagen, Denmark

K. B. Christensen · X. Fretté
Institute of Chemical Engineering, Biotechnology
and Environmental Technology, University of Southern
Denmark, Niels Bohrs Allé 1, 5230 Odense, Denmark

A. N. Assimopoulou · V. P. Papageorgiou
Organic Chemistry Laboratory, Department of Chemical
Engineering, Aristotle University of Thessaloniki,
54124 Thessaloniki, Greece

I. Kouskoumvekaki (✉)
Center for Biological Sequence Analysis, Department
of Systems Biology, Technical University of Denmark,
2800 Lyngby, Denmark
e-mail: irene@cbs.dtu.dk

Introduction

PPARs are fatty acid activated transcription factors that belong to the thyroid/retinoid nuclear receptor family. The α, δ, and γ subtypes of PPAR found in mammals, coordinate pathways involved in glucose and lipid homeostasis. PPARs are implicated in the pathology of various disease states including type II diabetes, obesity, dyslipidemia, atherosclerosis, neoplastic diseases and tumors, inflammatory conditions, and neurodegenerative diseases. PPARs are thus targets of numerous drug design and development efforts and the significant role of PPARs in disease treatment is the subject of many studies [1–4].

PPARγ is predominately expressed in adipose tissue and its significant role in lipid metabolism, adipogenesis, glucose homeostasis, and insulin sensitization is well documented [5–7]. Agonists for this subtype increase adipocyte differentiation and improve the storage of fatty acids. Furthermore, they enhance insulin sensitivity by a not fully understood mechanism that involves PPARγ activity in adipose tissue, skeletal muscle, macrophages, and liver.

Natural ligands of PPARγ are fatty acids as well as eicosanoids. Among a large variety of synthetic ligands are thiazolidinediones (TZDs) and some nonsteroidal anti-inflammatory drugs. TZDs have been used in clinical practice to treat type II diabetes for many years and have been shown to lower blood glucose levels and improve insulin sensitivity [8]. While the glucose lowering action of TZDs was well-known as early as 1988, it was not until 1995 that the nuclear receptor PPARγ was identified as their target and that its activation was shown to be responsible for their therapeutic benefits. However, despite their excellent potencies, administration of TZDs has been associated with severe side effects such as fluid retention, weight gain, cardiac hypertrophy, and hepatotoxicity [9, 10]. Troglitazone, for example, was withdrawn from therapeutic use due to liver toxicity and Farglitazar failed to pass phase III clinical trials due to the emergence of peripheral edema. Since October 2010, Rosiglitazone has been withdrawn from the European market after recommendations by the European Medicines Agency (EMA) and following concerns over excess cardiovascular risks. Though the drug is still available in the United States, the Food and Drug Administration (FDA) has stated that it should be restricted to patients who cannot be successfully treated with other medicine. Pioglitazone is currently in clinical practice despite being also linked to controversial side effects including an increased risk of cardiovascular related death. It is, thus, apparent that the search for PPARγ ligands with an improved mode of action is an important objective.

The occurrence of undesirable side effects has been linked to the use of TZDs that behave like full PPARγ agonists [12], where efficacy and side effects have been shown to be intrinsically linked. Higher efficacy compounds are associated with a greater propensity for side effects and increasing doses produce both greater benefits for glucose control as well as greater incidence and higher degrees of side effects. Thus, doses which would produce the maximal clinical benefit of PPARγ full agonists may not be tolerated by a significant number of patients and the full potential of PPARγ activation for insulin sensitization and glucose control may not be realized at approved clinical doses.

Partial PPARγ agonists, on the other hand, are ligands that upon binding to PPARγ provide diminished conformational stability of the receptor as opposed to full agonists, and thereby also recruit a different set of co-factors than the latter. The alternative co-factor usage is often reflected in suboptimal transcriptional activation of the receptor (defining the low efficacy properties of a partial agonist). It is generally recognized that the selective recruitment of co-factors in response to administration of a partial agonist does not induce the same magnitude of side-effects as observed for the full agonist TZDs [13]. It has, thus, been suggested that PPARγ partial agonists fulfill the requirements for beneficial PPARγ ligands, as they maintain their insulin sensitizing activity without having a strong adipogenic potential. On that account, partial agonists of PPARγ with decreased side effects on adipose tissue are investigated for their usability in the therapy of type II diabetes [14–16].

In this study we have combined in silico and in vitro approaches for the identification of natural compounds that act as PPARγ agonists. We have used pharmacophore-based Virtual Screening (VS) for the initial identification of target compounds and subsequently verified one of the hits using bioassay-guided chromatographic fractionation. VS is increasingly used as a cost-effective supplement to high-throughput screening and employs a range of methods for the in silico high-throughput assessment of large databases, and rapid evaluation and prioritization of compounds prior to wet-lab testing. Pharmacophore models in particular, involve the identification of the pharmacophoric pattern common to a set of known actives and the use of this pattern in a subsequent search. Pharmacophore-based virtual screening has only recently been used for the identification of novel ligands of PPARγ of synthetic [17, 18] or natural [19] origin. Experimentally validated PPARγ agonists from plants are hitherto few and include structurally diverse compounds from various sources such as fruits, vegetables, and medicinal plants [20–23]. Considering that plants have a long history in the traditional treatment of many diseases, including diabetes [24], natural product libraries represent a very promising source of novel PPARγ ligands.

Materials and computational method

Dataset of natural compounds

Virtual screening for PPARγ partial agonists was performed on the Chinese Natural Product Database [25] (CNPD v.2004.1). CNPD is a compilation of 57,346 compounds found in plants largely used in TCM (Traditional Chinese Medicine). These compounds originate from 2,611 plant species belonging to 457 different plant genera. After removal of salts, inorganic compounds and duplicates, we extracted 53,180 unique organic compounds in SDF format, which we imported into a Molecular Operating Environment (MOE) v. 2008.10 database [26]. These structures were washed, i.e. for all ionizable groups the correct protonation state at neutral pH conditions was produced, and energy minimized using the MMFF946 force field. Conformations were generated with a stochastic conformational search algorithm.

Partial agonist pharmacophore model

The partial agonist pharmacophore model was developed using the Pharmacophore Elucidator application of the MOE software. The Pharmacophore Elucidator generates a collection of pharmacophore queries from a collection of compounds some or all of which are active against a particular biological target such that all or most of active compounds satisfy the queries. The model was generated using the Unified scheme, based on a compound set of 13 PPARγ partial agonists, all selective to PPARγ; no dual or pan PPAR agonists were included (Table 1). The Unified scheme is the most comprehensive scheme available in MOE and contains 20 different annotation points (such as H-bond donor, h-bond acceptor, hydrophobe, etc.), covering atom, centroid and projected annotations. The pharmacophore was generated on the first aligned solution that was characterized by the highest accuracy and overlap. The model was subsequently used for the virtual screening of the CNPD database. To be considered as a hit, a compound had to fit all the features of the pharmacophore.

Docking of novel PPARγ partial agonist

Oleanonic acid was docked on the crystal structure 2F4B of PPARγ using MOE v. 2008.10. Prior to docking, all ligands and water were removed from the protein structure, hydrogens were added, ionization states were assigned, and tethered energy minimization was performed using the MMFF94 force field. The protonation state of oleanonic acid was calculated at physiological pH, with the carboxyl group deprotonated. Docking was performed with a rigid protein and a flexible ligand, using the Alpha PMI placement and the London dG scoring function.

For validation, EHS ([5-(3-{[6-(phenylcarbonyl)-1-propylnaphthalen-2-yl]oxy}propoxy)-1H-indol-1-yl]acetic acid), the ligand co-crystallized in the PDB complex with PPARγ, was used. The ligand re-docked in the same position as in the original complex with an RMSD of 1.43 Å. The interaction of the indole acid acetic group of the ligand with His449 is conserved, while the flexibility of the tail of the molecular structure is the one responsible for the deviation on the docking pose from the original in armII.

Fractionation of P. lentiscus oleoresin

HPLC-grade acetonitrile (MeCN) was obtained from Fischer Scientific (Leicestershire, UK). Formic acid (FA) 99%, trifluoro acetic acid (TFA) 99 + %, and chloroform-d (CDCl$_3$) 99.8 atom % D was obtained from Sigma–Aldrich (Steinheim, Germany). Fractions of P. lentiscus var. Chia oleoresin were isolated according to [38, 39].

Further sub-fractionation of the most active fractions 4 and 6 was carried out by Semi-preparative HPLC using a Dionex Ulti Mate 3000 pump equipped with a Dionex Ulti Mate Photodiode Array Detector (PDA) and a Develosil ODS-HG-5 RP-18 column (5 μm; 250 × 20 mm, Nomura Chemical Co.) as well as a Develosil ODS-HG-5 RP-18 pre-column (5 μm; 20 × 50 mm, Nomura Chemical Co.). Flow was 5 mL/min and injection volume was 2 mL. LC–MS data were obtained using a LTQ XL (ESI-2D-iontrap, Thermo Scientific) equipped with an Accela HPLC Pump and PDA Detector as well as an evaporative light scattering detector (ELSD, Sedex 80LT, SEDERE, Alfortville, France). Settings for the mass spectrometer were 36, 26, and 0 (arbitrary units) for sheath, auxillary, and sweep gas flow rates, respectively. Spray voltage was 5 kV, capillary temperature 275 °C, capillary voltage 3.10 V, tube lens 100 V, and AGC target settings were 3×10^4 and 1×10^4 for full MS and MS^n, respectively. Separations were obtained by the solvent gradient A = 1.00% FA in H$_2$O, B = 1.00% FA in MeCN; 0 min (35% B), 60 min (100% B), 70 min (100% B), 80 min (35% B), 90 min (35% B) on a Luna NH$_2$-column (5 μm; 250 × 4.6 mm, Phenomenex). Flow was 200 μL/min, temperature 35 °C, and injection volume 10 μL. 1D and 2D NMR data were acquired on a Bruker AVANCE III 400 MHz system using solvent signals (CDCl$_3$; δ_H 7.26/δ_C 77.7) as references.

The neutral fraction (sample 4, 100 mg) was further separated using semi-preparative HPLC by the solvent gradient A = 0.05% TFA in H$_2$O, B = MeCN; 0 min (50% B), 30 min (100% B), 80 min (100% B), 90 min (50% B), 95 min (50% B) to give four fractions 4-I (41 mg), 4-II (15.7 mg), 4-III (14.5 mg), and 4-IV (9.2 mg). The acidic fraction (sample 6, 100 mg) was likewise separated using semi-preparative HPLC by the solvent gradient A = 0.05% TFA in H$_2$O, B = MeCN; 0 min (60% B), 10 min (60% B), 40 min (100% B), 90 min (100% B), 100 min (60% B), 105 min (60% B) to give five fractions 6-I (17.3 mg), 6-II (6.6 mg), 6-III (12.3 mg), 6-IV (6.8 mg), and 6-V (41.1 mg).

Biological testing of P. lentiscus oleoresin fractions

PPARγ mediated transactivation

Mouse embryo fibroblasts (MEFs) were propagated in Dulbeccos Modified Eagle's Media (DMEM) supplemented with 10% fetal calf serum and antibiotics. MEFs were transfected in solution by Metafectene (Biontex) lipofection, essentially according to the manufacturer's instructions and seeded in DMEM supplemented with 10% fetal calf serum and antibiotics in 96-well dishes at 24,000 cells/cm^2. The transfection plasmid mix included the Gal4-responsive luciferase reporter, the expression

Table 1 Compound set of partial PPARγ agonists and their EC$_{50}$ values (μM) utilized for generation of the partial agonist pharmacophore model

Balaglitazone
EC$_{50}$ = 1.351 μM [27]
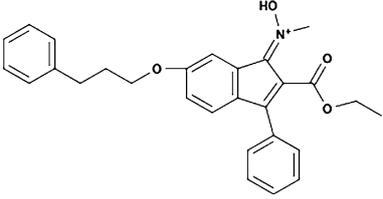
KR-62776
EC$_{50}$ = 0.05 μM [28]
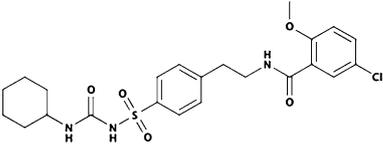
Glibenclamide
EC$_{50}$ = 7.6 μM [29]
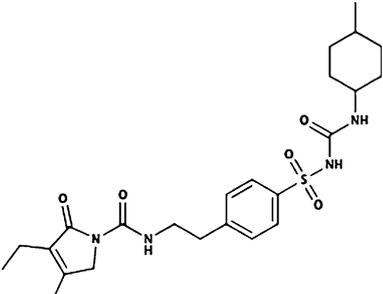
Glimepiride
EC$_{50}$ = 27 μM [29]
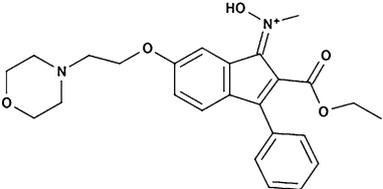
KR-62980
EC$_{50}$ = 0.015 μM [30]
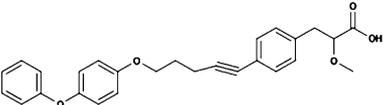
LSN862
EC$_{50}$ = 0.239 μM [31]
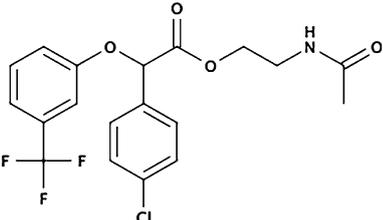
Halofenate
EC$_{50}$ = 30 μM [32]
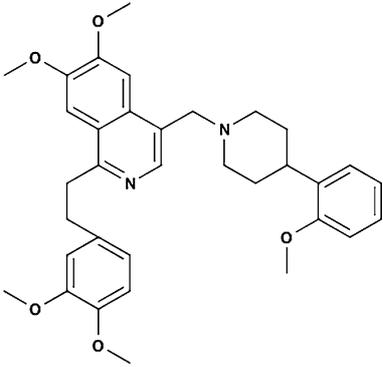
PA-082
EC$_{50}$ = 0.26 μM [33]
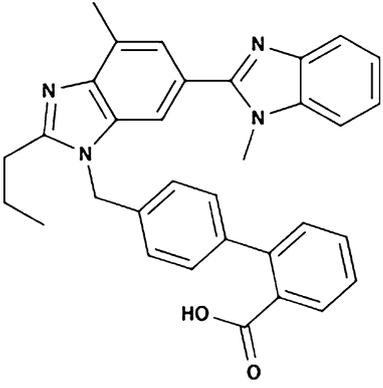
Telmisartan
EC$_{50}$ = 0.0045 μM [34]
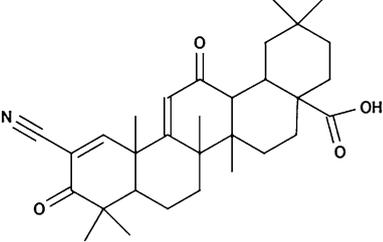
CDDO
EC$_{50}$ = 0.030 μM [35]
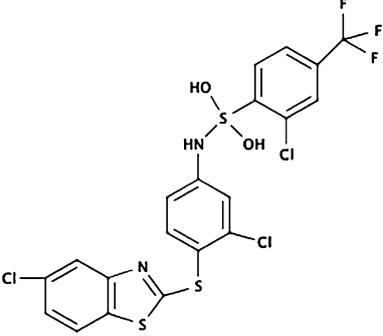
T2384
EC$_{50}$ = 0.56 μM [36]
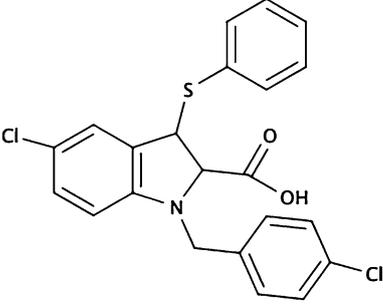
nTZDpa
EC$_{50}$ = 0.057 μM [13]

Table 1 continued
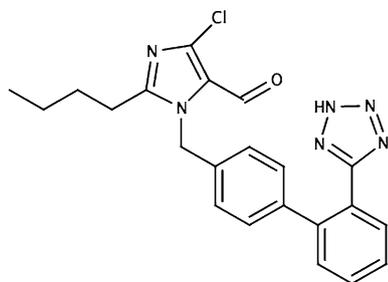
EXP-3179

$EC_{50} = 17.1 \ \mu M$ [37]

vector for the fusion between the Gal4 DNA-binding domain and the human PPARγ ligand binding domain, (Gal4(DBD)-hPPARγ(LBD)), and a CMV Renilla luciferase normalization vector (pRL-CMV, Promega). Six hours after addition of transfection mix to the cells, the media was changed to DMEM supplemented with vehicle (0.2% DMSO), positive control (1 μM Rosiglitazone) or compound (in competition assay cells were incubated with 0.3% DMSO or 0.3 μM Rosiglitazone and increasing concentrations of oleanonic acid as indicated). Approximately 18 h later, cells were harvested and lysates analyzed for Photinus and Renillaluciferase activity by luminometry. All experiments were performed in at least triplicate and each sample measured in duplicate. Luminometer raw data were analyzed in Microsoft Excel spreadsheets and presented as column graphs depicting average values of triplicates and including standard deviations.

Results and discussion

Pharmacophore-based virtual screening

A 4-point pharmacophore model was developed, which consists of one hydrogen bond acceptor, and three hydrophobic spheres (Fig. 1). The model matched all 13 partial agonists of PPARγ that were employed in the pharmacophore elucidation. The model's performance was validated on a recently discovered partial PPARγ agonist, Indeglitazar [40], which overlapped with the pharmacophore features

Fig. 1 4-Point pharmacophore model for PPARγ partial agonists superposed on Indeglitazar (RMSD: 0.50 Å). In MOE, pharmacophoric features are represented by a point encased in a sphere. The *spheres* depict the location tolerance allowed during virtual screening. Points not encased in spheres are other potential pharmacophore features on the Indeglitazar structure. *Blue* projected location of hydrogen-bond donor, 1 Å tolerance, *green* hydrophobic region, 1.4 Å tolerance, *orange* aromatic center, *red* CO2 centroid. On the *top left*, the 2D structure of Indeglitazar
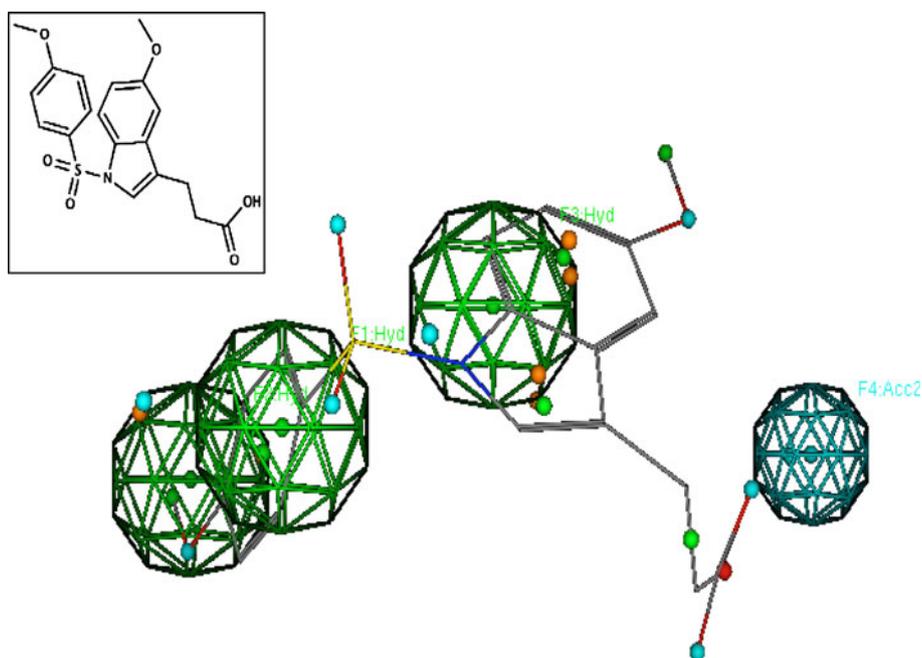
with a RMSD (Root Mean Square Deviation) = 0.5 Å. The hydrogen-bond acceptor projection (Acc2) feature of the pharmacophore model overlaps well with the propionic acid side chain of Indeglitazar, whereas the hydrophobic features of the model fall on the aromatic rings of the structure. According to Artis et al. [40], the carboxylate group of the propionic acid side chain forms hydrogen-bonds with residues His323, Tyr327, His449, and Tyr473.

In order to prioritize the best hits and reduce the number of false positives from the CNPD database, a volume constrain based on the union of volumes of the 13 partial agonists, as well as the Lipinski drug-like filter were applied. Pharmacophore-based screening of the CNPD database retrieved 939 compounds with RMSD ≤ 0.5 Å (see Supporting Information for list of chemical names and RMSD values).

A major bottleneck in virtual screening of natural compound libraries is the unavailability of the natural hits in pure form from commercial suppliers, combined with the costly, time-consuming and sometimes even unfeasible in vitro synthesis of the natural compound, or its isolation from a natural source. Within this framework, we examined carefully the obtained list of hits and selected methyl oleanonate (me-ester-3-oxo-olean-12-en-28-oic acid), ranked 24, for experimental validation of its predicted biological activity on PPARγ. Methyl oleanonate is a triterpene found in different species of *Pistacia*, some of them endemic to Europe. However, it is not the methyl ester itself that is present in the plant, but the acid,

oleanonic acid. This inconsistency in the CNPD is most likely due to the fact that GC–MS is primarily used for identification of these triterpenes and methylation is a necessary step in this technique for identification of acids. Oleanonic acid has primarily been identified as its methyl ester in the oleoresin from *Pistacia lentiscus* var. Chia [38, 39, 41] that is uniquely cultivated in southern Chios, a Greek island in the Aegean. Interestingly, in local traditional medicinal praxis, Chios mastic gum dispersed in water is used as antidiabetic medication [42].

Docking of oleanonic acid to PPARγ

In order to determine the putative binding mode and the potential ligand-target interactions involved in the binding of oleanonic acid to PPARγ, the former was docked on the Ligand Binding Domain (LBD) of the PDB (Protein Data Bank) entry 2F4B [43]. Figure 2 shows the best docking pose, where the carboxylic moiety of the compound forms hydrogen bonds with His323 and Tyr327 on helix 4/5 of armI, which is akin to the binding mode of Indeglitazar [40]. In addition, the remainder of the ligand is stabilized with several interactions in the hydrophobic pocket consisting of residues Gln286, Met364, Leu453 and Leu469. The fact that there are no predicted hydrogen bonding interactions between the carboxyl group and residues His449 and Tyr473 of the AF2 helix hints towards oleanonic acid being a partial PPARγ agonist, which was indeed our initial aim during the development of the pharmacophore model.

Fig. 2 Docking pose of oleanonic acid in the LBD of PPARγ, which shows that the carboxylic moiety of the compound forms hydrogen bonds with His323 and Tyr327 on helix 4/5 of armI. In addition, the remainder of the ligand is stabilized with several interactions in the hydrophobic pocket consisting of residues Gln286, Met364, Leu453 and Leu469
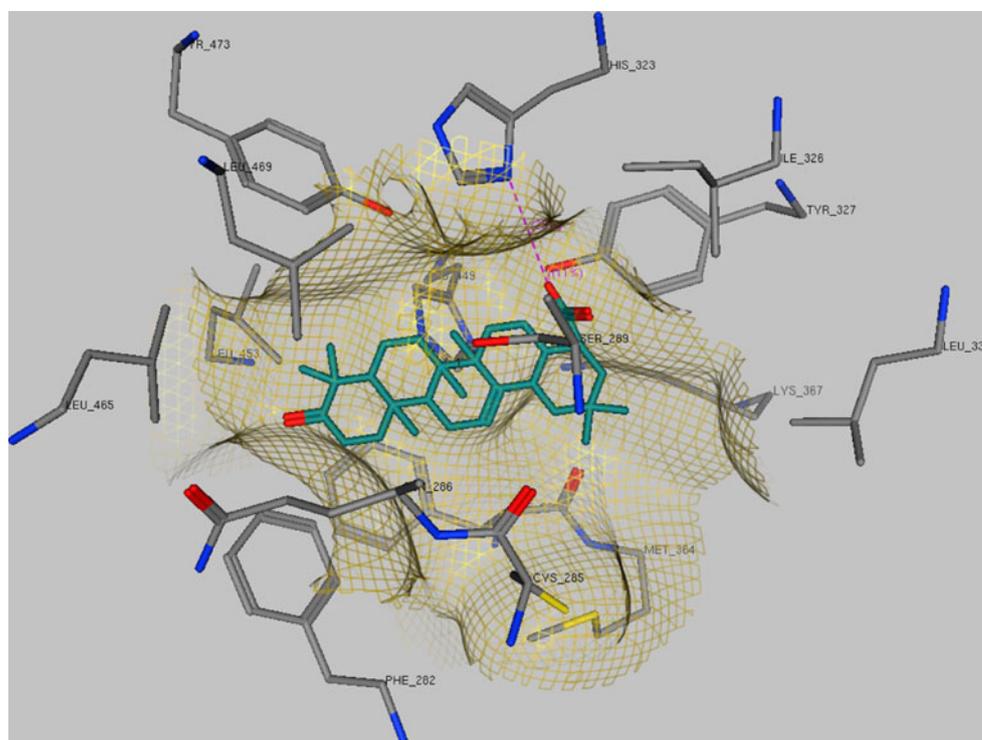
Pistacia lentiscus oleoresin; fractionation and biological testing

Mastic gum has been known from the time of Pedanius Dioscurides, (70ac) [11] and has been used in Greek folklore medicine for various gastrointestinal disorders such as gastralgia, dyspepsia, and peptic ulcer for more than 2,500 years. Furthermore, folklore medicine indicates that mastic gum components may be active against diabetes. Mastic gum has been reported to possess anti-inflammatory [44], antimicrobial [45], anticancer [44, 46, 47] as well as gastric and duodenal antiulcer activity [48, 49]. Consumption of mastic gum has also recently been associated with cardiovascular protection, where in vivo decrease in the cholesterol levels in the serum [50] and in vitro inhibition of oxidation of human low-density lipoproteins (LDL) [51] are reported. In previous studies, the oleoresin of *P. lentiscus* var. Chia was shown to exhibit antioxidant [52] and radical scavenging activities (Assimopoulou and Papageorgiou, unpublished data). The metabolites responsible for these reported bioactivities of *P. lentiscus* oleoresin have not been fully clarified. However, some studies on the biological activities of one of the major components, oleanonic acid do exist and include cytotoxic, uterotonic, and anti-Helicobacter activities [53–59]. In a recent report, Giner-Larza et al. [60] demonstrated the inflammatory activity of both oleanonic and oleanolic acid and suggested that the presence of the keto group in the former augments its anti-inflammatory activity in models related with the activation of 5-lipoxygenase. Investigations into the composition of *Pistacia lentiscus* oleoresin are generally based on examinations of its acidic and neutral fractions [38, 39, 41]. In the acidic fractions of *P. lentiscus* oleoresin, the triterpenes oleanonic acid, isomasticadienonic acid and masticadienonic acid are the major metabolites. In the neutral fraction, a much larger number of compounds are present but the dominating ones are 28-norolean-17-en-3-one and oleanonic aldehyde [38].

In the present study, six samples were initially isolated and analyzed for PPARγ activity, as listed in Table 2,

including the oleoresin of *P. lentiscus* var. Chia (sample 1) and its subfractions (samples 3–6), as well as the oleoresin of *P. terebinthus* var. Chia, a species very similar to *P. lentiscus* (sample 2). Although not all of these fractions contained oleanonic acid they were included in the experimental testing as many compounds structurally similar to oleanonic acid are present in these samples.

As seen from Fig. 3, fractions 4 and 6 possessed the ability to stimulate PPARγ-mediated transactivation and consequently they were selected for further fractionations. Fraction 6 was also the one with the highest content of oleanonic acid as observed from LC–MS (data not shown).

Separation of the major components present in the active sub-fractions was achieved using semi-preparative reversed phase HPLC. The neutral fraction (sample 4) of *P. lentiscus* oleoresin was separated into four fractions and the acidic fraction (sample 6) into five fractions. Testing of these fractions revealed the presence of PPARγ modulating activity in the sub-fractions 4-I, 6-I, and 6-II (Fig. 4).

LC–MS analysis showed that fraction 6-II almost exclusively contains oleanonic acid, supporting the pharmacophore model prediction for this molecule. LC–MS analysis of fractions 4-I and 6-I revealed that these fractions contain several compounds. However, as we in the present study focused on oleanonic acid, we did not examine which of these compounds were responsible for the observed activation of PPARγ. The acquired LC–MS data were compared to data from literature both from GC–

Table 2 Samples of oleoresin of *P. lentiscus* var. Chia, its subfractions and oleoresin of *P. terebinthus* var. Chia that were tested for their ability to activate PPARγ

Sample #	Sample
1	*P. lentiscus* oleoresin
2	*P. terebinthus* oleoresin*
3	Mastic oil
4	Neutral fraction
5	Acidic fraction I
6	Acidic fraction II

* Species similar to *P. lentiscus*
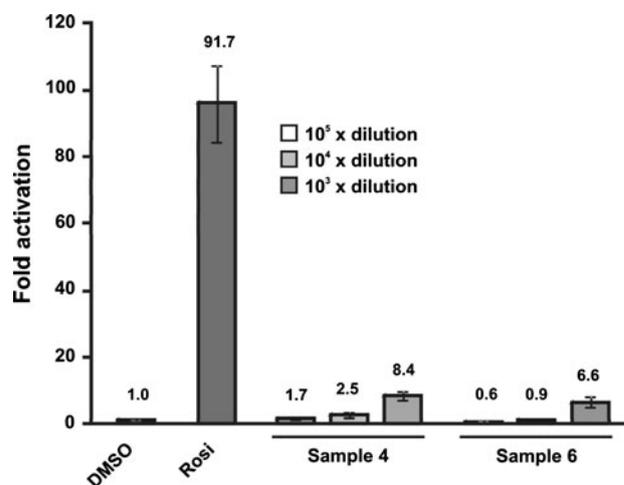
Fig. 3 PPARγ activating properties of *P. lentiscus* oleoresin neutral fraction (sample 4) and acidic fraction II (sample 6). In transient transfection experiments fractions of *P. lentiscus* oleoresin were able to induce transcriptional activation of a luciferase reporter driven by a fusion between the Gal4 DNA-binding domain and the human PPARγ ligand-binding domain (Gal4(DBD)-hPPARγ(LBD)). Shown is the relative activation compared to the DMSO vehicle, with each column representing the average ± standard deviation ($n \geq 3$) of a representative experiment. The PPARγ full agonist Rosiglitazone (Rosi) is included as a positive control for PPARγ agonist activation
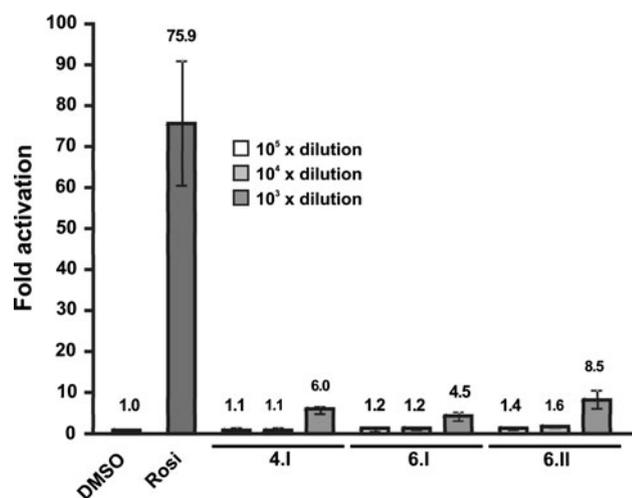
Fig. 4 PPARγ activating properties of *P. lentiscus* oleoresin sub-fractions 4.I, 6.I and 6.II. In transient transfection experiments fractions of *P. lentiscus* oleoresin were able to induce transcriptional activation of a luciferase reporter driven by Gal4 (DBD)-hPPARγ(LBD). Shown is the relative activation compared to the DMSO vehicle, with each column representing the average ± standard deviation ($n \geq 3$) of a representative experiment. The PPARγ full agonist Rosiglitazone (Rosi) is included as a positive control for PPARγ agonist activation

MS and LC–MS (1–3) and further verified by 1D and 2D NMR.

Further testing of oleanonic acid (fraction 6-II) data in dose response studies revealed that this compound acts as a low potency (mid μM range) PPARγ activator with an efficacy reaching app. 20% the activity of the full agonist Rosiglitazone at saturating concentration (Fig. 5).
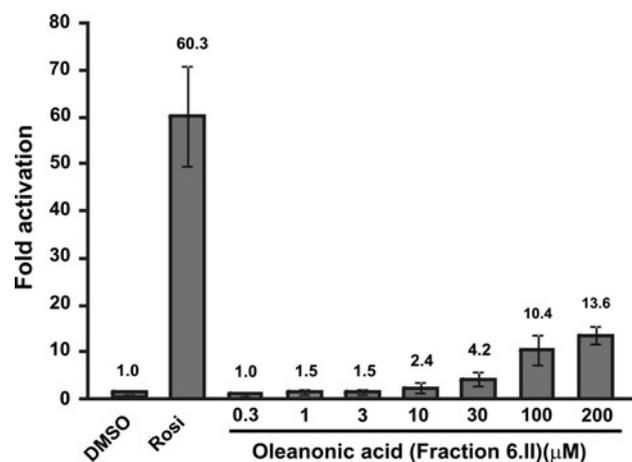

Fig. 5 Oleanonic acid induces transcriptional activation of PPARγ. In transient transfection experiments oleanonic acid dose dependently activates a luciferase reporter driven by the Gal4 (DBD)-hPPARγ(LBD). Shown is the relative activation compared to the DMSO vehicle, with each column representing the average ± standard deviation ($n \geq 6$) of a representative experiment. The PPARγ full agonist Rosiglitazone (Rosi) is included as a positive control for PPARγ agonist activation
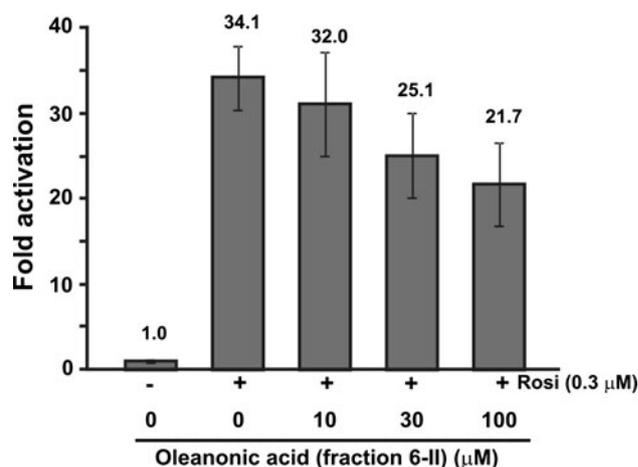

Fig. 6 Oleanonic acid antagonizes Rosiglitazone induced transcriptional activation of PPARγ. In transient transfection experiments oleanonic acid dose dependently attenuates Rosiglitazone (Rosi) induced PPARγ-mediated activation of a luciferase reporter. Shown is the relative activation compared to the DMSO vehicle, with each column representing the average ± standard deviation ($n \geq 6$) in a representative experiment

A competition assay demonstrated that oleanonic acid was able to dose dependently antagonize Rosiglitazone-mediated PPARγ transcriptional activation (Fig. 6), suggesting that oleanonic acid as predicted by the in silico screening may function as a partial agonist for PPARγ. Further analyses including determination of the ability of oleanonic acid to recruit co-factors will be required to corroborate whether oleanonic acid is a bona fide partial PPARγ agonist.

Conclusions

In summary, pharmacophore-based virtual screening of a library of natural compounds, in combination with bioassay-guided chromatographic fractionation, led to the successful identification of oleanonic acid as a new partial agonist for PPARγ. Oleanonic acid is found in the oleoresin of *Pistacia lentiscus* var. Chia (Chios mastic gum), which, according to local folk medicine, has antidiabetic properties. An often-observed situation among plant remedies, however, is pharmacokinetic synergy, where the pharmacological effect of the plant is the outcome of multiple active substances acting in combination towards the same or multiple biological targets [61]. In this work, we did observe that other compounds able to activate PPARγ were present in the tested fractions. Furthermore, activities towards other biological targets relevant to disease development should not be ruled out either. It is our intention to further investigate in a subsequent study the full activity profile of oleanonic acid and the other constituents of

P. lentiscus oleoresin towards a complete panel of biological targets related to type-2 diabetes.

References

1. Tobin JF, Freedman LP (2006) Nuclear receptors as drug targets in metabolic diseases: new approaches to therapy. Trends Endocrinol Metab 17(7):284–290

2. Chang F, Jaber LA, Berlie HD, O'Connell MB (2007) Evolution of peroxisome proliferator-activated receptor agonists. Ann Pharmacother 41(6):973–983

3. Wang Y (2010) Ppars: diverse regulators in energy metabolism and metabolic diseases. Cell Res 20(2):124–137

4. Cheatham W (2010) Peroxisome proliferator-activated receptor translational research and clinical experience. Am J Clin Nutr 91(1):262S–266S

5. Willson TM, Lambert MH, Kliewer SA (2001) Peroxisome proliferator-activated receptor γ and metabolic disease. Annu Rev Biochem 70:341–367

6. Semple RK, Chatterjee VKK, O'Rahilly S (2006) PPARγ and human metabolic disease. J Clin Invest 116(3):581–589

7. Higgins LS, Mantzoros CS (2008) The development of INT131 as a selective PPARγ modulator: approach to a safer insulin sensitizer. PPAR Res. doi:10.1155/2008/936906

8. Elte JWF, Blicklé JF (2007) Thiazolidinediones for the treatment of type 2 diabetes. Eur J Intern Med 18:18–25

9. Guan Y, Hao C, Cha DR, Rao R, Lu W, Kohan DE, Magnuson MA, Redha R, Zhang Y, Breyer MD (2005) Thiazolidinediones expand body fluid volume through PPARγ stimulation of enac-mediated renal salt absorption. Nat Med 11:861–866

10. Pan HJ, Lin Y, Chen YE, Vance DE, Leiter EH (2006) Adverse hepatic and cardiac responses to rosiglitazone in a new mouse model of type 2 diabetes: relation to dysregulated phosphatidylcholine metabolism. Vasc Pharmacol 45:65–71

11. Wellman M (ed) (1958) Dioscurides P. In de materia medica. Berloni Apud Weidmannos, Berlin

12. Barroso I, Gurnell M, Crowley VE, Agostini M, Schwabe JW, Soos MA, Maslen GL, Williams TD, Lewis H, Schafer AJ, Chatterjee VK, O'Rahilly S (1999) Dominant negative mutations in human PPARγ associated with severe insulin resistance, diabetes mellitus and hypertension. Nature 402:880–883

13. Berger J, Petro AE, Macnaul KL, Kelly LJ, Zhang BB, Richards K, Elbrecht A, Johnson BA, Zhou G, Doebber TW, Biswas C, Parikh M, Sharma N, Tanen MR, Thompson GM, Ventre J, Adams AD, Mosley R, Surwit RS, Moller DE (2003) Distinct properties and advantages of a novel peroxisome proliferator-activated protein [gamma] selective modulator. Mol Endocrinol 17(4):662–676

14. Lu I-L, Huang C-F, Peng Y-H, Lin Y-T, Hsieh H-P, Chen C-T, Lien T-W, Lee H-J, Mahindroo N, Prakash E, Yueh A, Chen H-Y, Goparaju CMV, Chen X, Liao C-C, Chao Y-S, Hsu JT-A, Wu S-Y (2006) Structure-based drug design of a novel family of pparγ partial agonists: Virtual screening, x-ray crystallography, and in vitro/in vivo biological activities. J Med Chem 49:2703–2712

15. Pochetti G, Godio C, Mitro N, Caruso D, Galmozzi A, Scurati S, Loiodice F, Fracchiolla G, Tortorella P, Laghezza A, Lavecchia A, Novellino E, Mazza F, Crestan M (2007) Insights into the mechanism of partial agonism. J Biol Chem 282(23):17314–17324

16. Towfighi A, Ovbiagele B (2008) Partial peroxisome proliferator-activated receptor agonist angiotensin receptor blockers. Potential multipronged strategy in stroke prevention. Cerebrovasc Dis 26(2):106–112

17. Markt P, Schuster D, Kirchmair J, Laggner C, Langer T (2007) Pharmacophore modeling and parallel screening for PPAR ligands. J Comput Aided Mol Des 21:575–590

18. Markt P, Petersen RK, Flindt EN, Kristiansen K, Kirchmair J, Spitzer G, Distinto S, Schuster D, Wolber G, Laggner C, Langer T (2008) Discovery of novel ppar ligands by a virtual screening approach based on pharmacophore modeling, 3d shape, and electrostatic similarity screening. J Med Chem 51:6303–6317

19. Tanrikulu Y, Rau O, Schwarz O, Proschak E, Siems K, Müller-Kuhrt L, Schubert-Zsilavecz M, Schneider G (2009) Structure-based pharmacophore screening for natural-product-derived pparg agonists. Chem Biol Chem 10:75–78

20. Huang TH, Kota BP, Razmovski V, Roufogalis BD (2005) Herbal or natural medicines as modulators of peroxisome proliferator-activated receptors and related nuclear receptors for therapy of metabolic syndrome. Basic Clin Pharmacol Toxicol 96:3–14

21. Christensen KB, Minet A, Svenstrup H, Grevsen K, Zhang H, Schrader E, Rimbach G, Wein S, Wolffram S, Kristiansen K, Christensen LP (2009) Identification of plant extracts with potential antidiabetic properties: Effect on human peroxisome proliferator-activated receptor (PPAR), adipocyte differentiation and insulin- stimulated glucose uptake. Phytother Res 23(9):1316–1325

22. Christensen K, Petersen R, Petersen S, Kristiansen K, Christensen L (2009) Activation of PPARgamma by metabolites from the flowers of purple coneflower (*Echinacea purpurea*). J Nat Prod 22(72):933–937

23. Christensen K, Petersen R, Kristiansen K, Christensen L (2010) Identification of bioactive compounds from flowers of black elder (*Sambucus nigra* L.) that activate the human peroxisome proliferator-activated receptor (PPAR) gamma. Phytother Res 24(Suppl 2):S129–S132

24. Marles RJ, Farnsworth NR (1995) Antidiabetic plants and their active constituents. Phytomedicine 2:137–189

25. Shen J, Xu X, Cheng F, Liu H, Luo X, Shen J, Chen K, Zhao W, Shen X, Jiang H (2003) Virtual screening of natural products for discovering active compounds and target information. Curr Med Chem 10:2327–2342

26. Molecular operating environment, v.2008.10 (2008) Chemical computing group. Montreal, Quebec

27. Larsen PJ, Lykkegaard K, Larsen LK, Fleckner J, Sauerberg P, Wassermann K, Wulff EM (2008) Dissociation of antihyperglycaemic and adverse effects of partial perioxisome proliferator-activated receptor (PPAR-gamma) agonist balaglitazone. Eur J Pharmacol 596(1–3):173–179

28. Kim J, Han DC, Kim JM, Lee SY, Kim SJ, Woo JR, Lee JW, Jung SK, Yoon KS, Cheon HG, Kim SS, Hong SH, Kwon BM (2009) PPAR gamma partial agonist, KR-62776, inhibits adipocyte differentiation via activation of erk. Cell Mol Life Sci 66(10):1766–1781

29. Fukuen S, Iwaki M, Yasui A, Makishima M, Matsuda M, Shimomura I (2005) Sulfonylurea agents exhibit peroxisome proliferator-activated receptor gamma agonistic activity. J Biol Chem 280(25):23653–23659

30. Kim KR, Lee JH, Kim SJ, Rhee SD, Jung WH, Yang SD, Kim SS, Ahn JH, Cheon HG (2006) KR-62980: a novel peroxisome proliferator-activated receptor gamma agonist with weak adipogenic effects. Biochem Pharmacol 72(4):446–454

31. Reifel-Miller A, Otto K, Hawkins E, Barr R, Bensch WR, Bull C, Dana S, Klausing K, Martin JA, Rafaeloff-Phail R, Rafizadeh-Montrose C, Rhodes G, Robey R, Rojo I, Rungta D, Snyder D, Wilbur K, Zhang T, Zink R, Warshawsky A, Brozinick JT (2005) A peroxisome proliferator-activated receptor alpha/gamma dual agonist with a unique in vitro profile and potent glucose and lipid effects in rodent models of type 2 diabetes and dyslipidemia. Mol Endocrinol 19(6):1593–1605

32. Allen T, Zhang F, Moodie SA, Clemens LE, Smith A, Gregoire F, Bell A, Muscat GE, Gustafson TA (2006) Halofenate is a selective peroxisome proliferator-activated receptor gamma modulator with antidiabetic activity. Diabetes 55(9):2523–2533

33. Burgermeister E, Schnoebelen A, Flament A, Benz J, Stihle M, Gsell B, Rufer A, Ruf A, Kuhn B, Märki HP, Mizrahi J, Sebokova E, Niesor E, Meyer M (2006) A novel partial agonist of peroxisome proliferator-activated receptor-gamma (PPAR-gamma) recruits PPARgamma-coactivator-1alpha, prevents triglyceride accumulation, and potentiates insulin signaling in vitro. Mol Endocrinol 20(4):809–830

34. Benson SC, Pershadsingh HA, Ho CI, Chittiboyina A, Desai P, Pravenec M, Qi N, Wang J, Avery MA, Kurtz TW (2004) Identification of telmisartan as a unique angiotensin ii receptor antagonist with selective ppargamma-modulating activity. Hypertension 43(5):993–1002

35. Wang Y, Porter WW, Suh N, Honda T, Gribble GW, Leesnitzer LM, Plunket KD, Mangelsdorf DJ, Blanchard SG, Willson TM, Sporn MB (2000) A synthetic triterpenoid, 2-cyano-3, 12-di-oxooleana-1, 9-dien-28-oic acid (CDDO), is a ligand for the peroxisome proliferator-activated receptor gamma. Mol Endocrinol 14(10):1550–1556

36. Li Y, Wang Z, Furukawa N, Escaron P, Weiszmann J, Lee G, Lindstrom M, Liu J, Liu X, Xu H, Plotnikova O, Prasad V, Walker N, Learned RM, Chen JL (2008) T2384, a novel antidiabetic agent with unique peroxisome proliferator-activated receptor gamma binding properties. J Biol Chem 283(14):9168–9176

37. Schupp M, Lee LD, Frost N, Umbreen S, Schmidt B, Unger T, Kintscher U (2006) Regulation of peroxisome proliferator-activated receptor gamma activity by losartan metabolites. Hypertension 47(3):586–589

38. Assimopoulou AN, Papageorgiou VP (2005) GC-MS analysis of penta- and tetra-cyclic triterpenes from resins of *Pistacia* species. Part i. *Pistacia lentiscus* var. Chia. Biomed Chromatogr 19:285–311

39. Assimopoulou AN, Ganzera M, Stuppner H, Papageorgiou VP Determination of penta- and tetra- cyclic triterpenes in *Pistacia lentiscus* resin. In: 57th international congress and annual meeting of the Society for Medicinal Plant and Natural Product Research, Geneva, 2009. Planta Medica 75(9)

40. Artis DR, Lin JJ, Zhang C, Wang W, Mehra U, Perreault M, Erbe D, Krupka HI, England BP, Arnold J, Plotnikov AN, Marimuthu A, Nguyen H, Will S, Signaevsky M, Kral J, Cantwell J, Settachatgull C, Yan DS, Fong D, Oh A, Shi S, Womack P, Powell B, Habets G, West BL, Zhang KYJ, Milburna MV, Vlasuk GP, Hirth KP, Nolop K, Bollag G, Ibrahim PN, Tobin JF (2009) Scaffold-based discovery of indeglitazar, a PPAR pan-active anti-diabetic agent. PNAS 106:262–267

41. Papageorgiou VP, Bakola-Christianopoulou MN, Apazidou KK, Psarros EE (1997) Gas chromatographic-mass spectroscopic analysis of the acidic triterpenic fraction of mastic gum. J Chromatogr A 769:263–273

42. http://www.shape.gr/news/153/ARTICLE/1980/2009-06-22.html. Accessed May 04, 2010

43. Mahindroo N, Wang CC, Liao CC, Huang CF, Lu IL, Lien TW, Peng YH, Huang WJ, Lin YT, Hsu MC, Lin CH, Hsu JT, Chen X, Lyu PC, Chao YS, Wu SY, Hsieh HP (2006) PDB id: 2f4b

44. Duke J (1983) Medicinal plants of the bible, vol 90. Trado-medic books, New York

45. Magiatis P, Melliou E, Skaltsounis AL, Chinou IB, Mitaku S (1999) Chemical composition and antimicrobial activity of the essential oil of *Pistacia lentiscus* var. Chia. Planta Med 65:749–752

46. Hartwell J (1967) Plants used against cancer. Lloydia 30(4):395–397

47. Doi K, Wei M, Kitano M, Uematsu N, Inoue M, Wanibuchi H (2009) Enhancement of preneoplastic lesion yield by chios mastic gum in a rat liver medium-term carcinogenesis bioassay. Toxicol Appl Pharmacol 234(1):135–142

48. Al-Said M, Ageel AM, Parmar NS, Tariq M (1986) Evaluation of mastic obtained from *Pistacia lentiscus* crude drug for gastric and duodenal anti-ulcer activity. J Ethnopharmacol 15:271–278

49. Dabos KJ, Sfika E, Vlatta LJ, Frantzi D, Amygdalos GI, Giannikopoulos G (2010) Is chios mastic gum effective in the treatment of functional dyspepsia? A prospective randomised double-blind placebo controlled trial. J Ethnopharmacol 127(2):205–209

50. Triantafyllou A, Chaviaras N, Sergentanis TN, Protopapa E, Tsaknis J (2007) Chios mastic gum modulates serum biochemical parameters in a human population. J Ethnopharmacol 111(1):43–49

51. Andrikopoulos NK, Kaliora AC, Assimopoulou AN, Papapeorgiou VP (2003) Biological activity of some naturally occurring resins, gums and pigments against *in vitro* LDL oxidation. Phytother Res 17:501–507

52. Assimopoulou AN, Zlatanos SN, Papageorgiou VP (2005) Antioxidant activity of natural resins and bioactive triterpenes in oil substrates. Food Chem 92:721–727

53. Sewram V, Raynor W, Mulholland DA, Raidoo DM (2000) The uterotonic activity of compounds isolated from the supercritical fluid extract of *Ekebergia capensis*. Pharm Biomed Anal 24(1):133–145

54. Sewram V, Raynor MW, Raidoo DM, Mulholland DA (1998) Coupling safe to uterotonic bioassay: an on-line approach to analysing medicinal plants. J Pharm Biomed Anal 18(3):305–318

55. Chiang YM, Chang JY, Kuo CC, Chang CY, Kuo YH (2005) Cytotoxic triterpenes from the aerial roots of Ficus microspora. Phytochemistry 66:495–501

56. Rui L, Qian-Qun G, Cheng-Bin C, Bing C, Hong-Bing L, Lei W, Hua-Shi G (2005) 12α, 13-dihydroxyolean-3-oxo-28-oic acid, a new triterpene, and the known oleanonic acid as a new cell cycle inhibitor from *Schefflera venulosa*. Chin J Chem 23:242–244

57. Nguyen AT, Fontaine J, Malonne H, Claeys M, Luhmer M, Duez P (2005) A sugar ester and an iridoid glycoside from *Scrophularia ningpoensis*. Phytochemistry 66(19):1186–1191

58. Paraschos S, Magiatis P, Mitakou S, Petraki K, Kalliaropoulos A, Maragkoudakis P, Mentis A, Sgouras D, Skaltsounis AL (2007) *In vitro* and *in vivo* activities of chios mastic gum extracts and constituents against *Helicobacter pylori*. Antimicrob Agents Chemother 51(2):551–559

59. Bona SG, Bono L, Daghetta L, Marone P (2001) Bactericidal activity of *Pistacia lentiscus* gum mastic against *Helicobacter pylori*. J Chemother 13(6):611–614

60. Giner-Larza EM, Mañez S, Recio MC, Giner RM, Prieto JM, Cerdá-Nicolás M, Ríos JL (2001) Oleanonic acid, a 3-oxotriter-pene from *Pistacia*, inhibits leukotriene synthesis and has anti-inflammatory activity. Eur J Pharmacol 428:137–143

61. Nahrstedt A, Butterweck V (2010) Lessons learned from herbal medicinal products: the example of St. John's wort. J Nat Prod [Epub ahead of print]