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Dehydrogenase 1

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Pistacia lentiscus Oleoresin: Virtual Screening and Identification of Masticadienonic and Isomasticadienonic Acids as Inhibitors of 11 β -Hydroxysteroid Dehydrogenase 1

Authors

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Key words

- *Pistacia lentiscus*
- Anacardiaceae
- 11 β -hydroxysteroid dehydrogenase
- virtual screening
- antidiabetic
- diabetes

Abstract



In traditional medicine, the oleoresinous gum of *Pistacia lentiscus* var. *chia*, so-called mastic gum, has been used to treat multiple conditions such as coughs, sore throats, eczema, dyslipidemia, and diabetes. Mastic gum is rich in triterpenes, which have been postulated to exert antidiabetic effects and improve lipid metabolism. In fact, there is evidence of oleanonic acid, a constituent of mastic gum, acting as a peroxisome proliferator-activated receptor γ agonist, and mastic gum being antidiabetic in mice *in vivo*. Despite these findings, the exact antidiabetic mechanism of mastic gum remains unknown. Glucocorticoids play a key role in regulating glucose and fatty acid metabolism, and inhibition of 11 β -hydroxysteroid dehydrogenase 1 that converts inactive cortisone to active cortisol has been proposed as a promising approach to combat metabolic disturbances including diabetes. In this study, a pharmacophore-based virtual screening was applied to filter a natural product database for possible 11 β -hydroxysteroid dehydrogenase 1 inhibitors. The hit list analysis was especially focused on the triterpenoids present in *Pistacia* species. Multiple triterpenoids, such as masticadienonic acid and isomasticadienonic acid, main constituents of

mastic gum, were identified. Indeed, masticadienonic acid and isomasticadienonic acid selectively inhibited 11 β -hydroxysteroid dehydrogenase 1 over 11 β -hydroxysteroid dehydrogenase 2 at low micromolar concentrations. These findings suggest that inhibition of 11 β -hydroxysteroid dehydrogenase 1 contributes to the antidiabetic activity of mastic gum.

Abbreviations



AR:	aromatic ring
11 β -HSD:	11 β -hydroxysteroid dehydrogenase
GR:	glucocorticoid receptor
H:	hydrophobic area
HBA:	hydrogen bond acceptor
HBD:	hydrogen bond donor
H6PDH:	hexose-6-phosphate dehydrogenase
M:	metal binding area
MR:	mineralocorticoid receptor
NI:	negative ionizable group
PDB:	Protein Data Bank
PI:	positive ionizable group
PPAR γ :	peroxisome proliferator-activated receptor γ
XVOL:	exclusion volume

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Introduction



Pistacia lentiscus var. *chia* (Anacardiaceae family) is a tree that grows exclusively on the Greek island Chios. It is mainly exploited for its oleoresinous gum, the so-called mastic gum [1]. This oleoresin is harvested in a traditional way by longitudinal incisions from the tree as tears or droplets, although an alternative technique called liquid collection has also been applied. In the latter method, the stimulating agent ethrel is used for resin excretion after incision of the tree to in-

crease the mastic gum productivity. The gum harvested by this liquid method is produced in fluid form and has a characteristic odor [2]. In addition to controlling cholesterol levels and combating diabetes, the oleoresin has been used for centuries against coughs, sore throats, eczema, stomachaches, kidney stones, pain, and rheumatism [3].

The medicinal effects of *P. lentiscus* are proposed to be caused by the secondary metabolite triterpenes that are found at high concentrations in both the acidic and neutral fractions of *Pistacia*

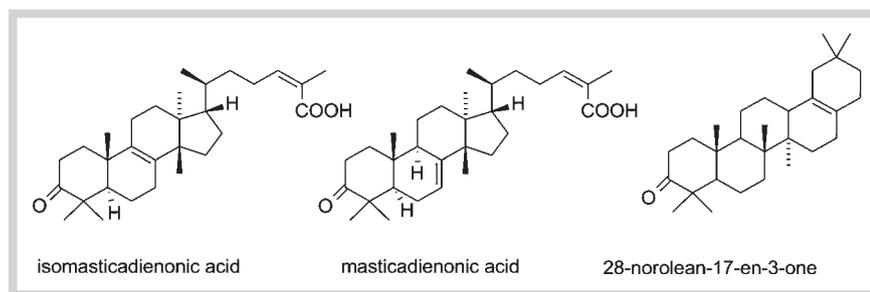


Fig. 1 Main constituents of *P. lentiscus* oleoresin (mastic gum).

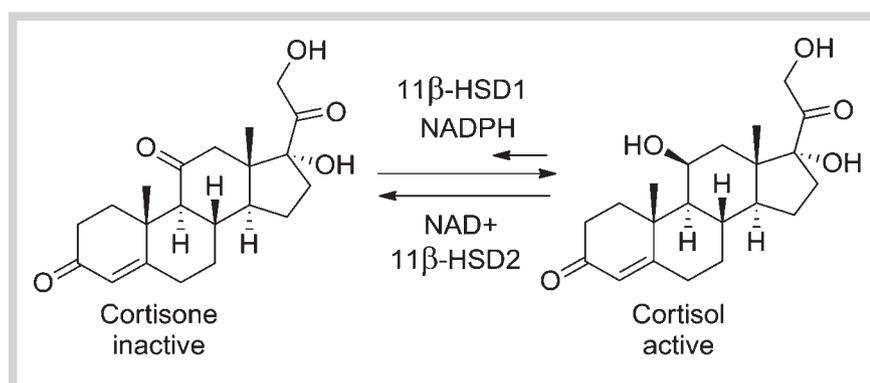


Fig. 2 Interconversion of cortisone to cortisol and vice versa by 11β-hydroxysteroid dehydrogenases.

oleoresins. Generally, the triterpenes are derivatives from 11 skeletons: Δ12-oleanene, Δ18-oleanene, 28-nor-Δ17-oleanene, Δ7-tirucallene, 24,25-dehydro-Δ7-tirucallene, Δ8-tirucallene, 24,25-dehydro-Δ8-tirucallene, dammarane, lupane, lupene, and Δ12-lupene [2]. The concentrations of different triterpenes vary with *Pistacia* species and the resin harvest method. The resin from *P. lentiscus* var. *chia* was found to contain 36 triterpenes when harvested in a traditional way and in the case of liquid harvesting, 26 different triterpenes were found [4]. The main constituents in both harvest methods are isomasticadienonic acid, masticadienonic acid, and 28-norolean-17-en-3-one (◻ Fig. 1).

In traditional medicine, mastic gum has been used against diabetes, a complex condition where the organism does not respond normally to the absorbed glucose. This is caused either by decreased insulin production (type 1 diabetes) or by insulin resistance, impaired glucose intake, and gluconeogenesis (type 2 diabetes), leading to elevated blood glucose levels [5]. Modern Western medicine combats diabetes by direct insulin injections, insulin sensitizing therapy, or enhancing the insulin secretion. Except for direct insulin injections, the treatment of diabetes involves multiple targets that play a role in glucose intake, gluconeogenesis, insulin sensitivity, and insulin secretion [5]. Among these targets is PPAR γ , which enhances insulin sensitivity and fatty acid storage upon activation [6]. In fact, oleanonic acid, a constituent of mastic gum, has been shown to activate PPAR γ [7]. An *in vivo* study in rats indicated that daily consumption of mastic gum resulted in a 40% decrease of blood glucose levels in high-fat diet-fed rats that also received streptozotocin injection according to a protocol that induced diabetes II [8]. In addition, the administration of mastic gum decreased blood glucose levels and improved serum fatty acid levels in diabetic mice [9]. Nevertheless, it is likely that these effects are not only mediated through PPAR γ . Thus, to understand the *in vivo* effects of mastic gum, the compounds involved and the underlying mechanisms need to be explored.

One possible explanation for the antidiabetic effects of mastic gum could be an interference with glucocorticoid metabolism. Glucocorticoids regulate carbohydrate and fat metabolism by decreasing glucose uptake and utilization, as well as increasing gluconeogenesis in the liver [10]. By affecting lipolysis and fat distribution, glucocorticoids are associated with the development of dyslipidemia that is often related to type 2 diabetes, metabolic disorders, and Cushing's syndrome. Glucocorticoids act via GRs and MRs, and their intracellular, pre-receptor concentrations are dependent on 11β-HSDs (◻ Fig. 2) [11]. 11β-HSD1 converts cortisone to its active hydroxyl derivative cortisol. This enzyme uses NADPH as a cofactor and the limiting factor of its cortisone-reducing activity is the coexpression with H6PDH that regenerates NADPH from NADP⁺ [12, 13]. Therefore, *in vivo*, 11β-HSD1 acts predominantly as a reductase. In contrast, 11β-HSD2 is an NAD⁺-dependent dehydrogenase responsible for the oxidative inactivation of cortisol to cortisone [14]. 11β-HSD1 is highly expressed in the liver, adrenals, adipose tissue, and skeletal muscles [15], whereas 11β-HSD2 is found in the kidneys, colon, and placenta [11, 16].

Since circulating cortisol levels (corticosterone in rodents) affect glucose and lipid metabolism, 11β-HSD1 is considered a promising intervention point to treat type 2 diabetes and metabolic disorders [17, 18]. In fact, this hypothesis is supported by biological data; the overexpression of 11β-HSD1 in adipose tissue in mice has been shown to cause visceral obesity, hyperglycemia, insulin resistance, and increased serum fatty acid and triglyceride levels [19]. Additionally, high corticosterone concentrations found only in the liver do not cause obesity or central adiposity, but instead cause steatosis, dyslipidemia, hypertension, and mild insulin resistance [20]. 11β-HSD1 knockout mice were shown to have an increased adrenal corticosterone production as a result of the impaired hepatic regeneration of active glucocorticoids, and they resisted obesity- or stress-related hyperglycemia [21]. Moreover, 11β-HSD1 inhibitors have been shown to improve several meta-

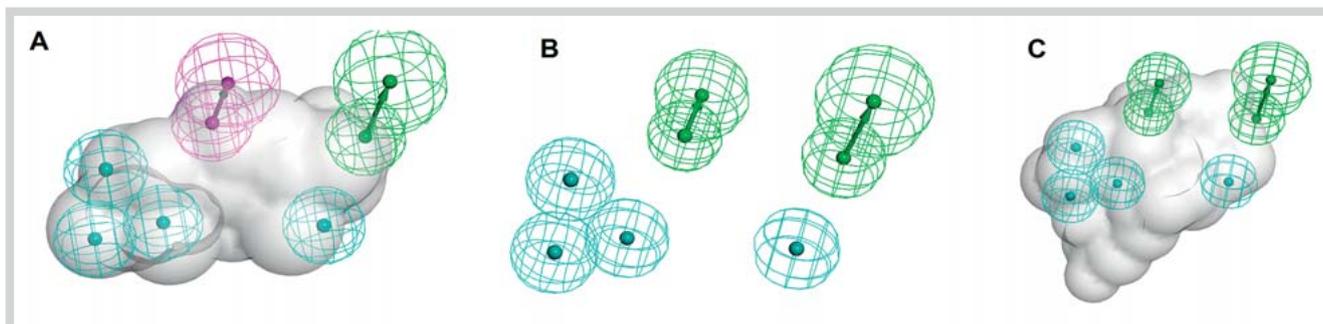


Fig. 3 Pharmacophore models for 11β -hydroxysteroid dehydrogenase 1 inhibition. The original model (A), intermediate refined model (B), and the final refined model that was used for virtual screening (C). The pharmacophore

features are color-coded: hydrophobic – cyan, hydrogen bond donor – magenta, hydrogen bond acceptor – green, shape – gray. (Color figure available online only.)

bolic parameters as well as atherosclerosis in mice by decreasing aortic lesions [17,22]. Thus, 11β -HSD1 inhibitors constitute a promising way to treat metabolic syndrome and type 2 diabetes in rodent models. However, when inhibiting 11β -HSD1, selectivity over 11β -HSD2 is important to avoid adverse effects such as severe hypokalemia, hypertension, edema formation, and renal enlargement, which are all consequences of cortisol-dependent MR activation [23].

There is a large number of known 11β -HSD1 inhibitors, mostly small synthetic chemicals [24–26]. In addition, compounds from natural origin have been shown to inhibit 11β -HSD1. Most of these natural compounds are triterpenes, such as corosolic acid, ursolic acid, glycyrrhethinic acid, and its derivatives [27,28]. Because mastic gum is rich in triterpenes, the inhibition of 11β -HSD1 could be one explanation for the observed antidiabetic effects of mastic gum. To test this hypothesis, a virtual screening campaign to search for 11β -HSD1 inhibitors from natural origin was launched. In virtual screening, a database of compounds is filtered to match the query requirements, and it has been considered a suitable tool for setting biological testing priorities also in the natural products field [29,30]. One common way to perform virtual screening is the pharmacophore-based method. In this method, pharmacophore models representing the 3D arrangement of those electrostatic and steric functionalities that make the small molecule active towards its target protein [31] are used as a filter. Pharmacophore models consist of features such as hydrogen bond acceptor (HBA)/hydrogen bond donor (HBD), hydrophobic areas (H), aromatic rings (AR), positively ionizable (PI) and negatively ionizable (NI) groups, as well as metal binding areas (M). Exclusion volumes (XVOLs) – forbidden areas – or a shape can be added to mimic the size and the shape of the binding pocket or active ligands. The result of a virtual screening is a so-called hit list that contains those compounds which chemical functionalities match with the features of the query pharmacophore. These compounds are predicted to be active towards the target.

To support drug development and to discover new 11β -HSD1 inhibitors, a pharmacophore model for 11β -HSD1 inhibition has been developed and reported [32]. This pharmacophore model consisted of six chemical features: four Hs, one HBA, one HBD, and a shape (Fig. 3A). The model was theoretically and experimentally successfully validated and used for virtual screening campaigns. However, during the recent years, new 11β -HSD1 inhibitors have been rapidly reported, and the pharmacophore model needed improvement to ensure the best performance.

Therefore, it was refined according to the newly published 11β -HSD1 inhibitors to better represent the current state of knowledge. First, the HBD function of the original model was exchanged with an HBA, and the shape restriction was removed (Fig. 3B). However, this model was not very restrictive, and therefore, as a further refinement step, a new shape restriction was added (Fig. 3C) [33]. This refined model, which was named model 4_{new} in its original publication [33], was employed for the virtual screening of a natural compound database to test the theory of *P. lentiscus* oleoresin constituents as 11β -HSD1 inhibitors prior to *in vitro* testing.

Results

In order to search for natural compounds with antidiabetic activity, especially focusing on the constituents of *P. lentiscus*, a pharmacophore-based virtual screening of the DIOS natural product database was performed. The DIOS database is an in-house database comprising 9676 secondary metabolites from 800 medicinal plants described by Dioscorides in his *De Materia Medica* [34]. The refined 11β -HSD1 model (Fig. 3C) returned 305 hits from the virtual screening. The hit list contained 155 terpenes, including 96 triterpenes, among which 27 were from *Pistacia* species and 8 were *P. lentiscus* constituents (Fig. 4). Other frequent classes were lipids and flavonoids with 30 and 28 compounds, respectively. Mostly, the triterpenes present in *Pistacia* species were derivatives of masticadienonic and isomasticadienonic acids. The focus of the biological evaluation was set on the whole resin, its acidic fraction containing mainly the above triterpenes, and on purified compounds. Among the eight virtual hits obtained from the species *P. lentiscus*, almost all of them belong to the acidic fraction that was isolated from *P. lentiscus* var. *chia* oleoresin. The two main constituents masticadienonic acid and isomasticadienonic acid were chosen for biological evaluation. The other virtual hits, masticadienolic acid and oleanolic acid, have previously been reported as constituents of *P. lentiscus* var. *chia* [35]; however, they were not detected in the resin batch that was used for biological evaluation [4] and could therefore not be tested. The remaining four hits were excluded because these were not constituents of the *P. lentiscus* var. *chia*, although they were commonly found in *P. lentiscus* L.

After harvesting and isolating the substances of interest, their inhibitory activity against 11β -HSD1 and 11β -HSD2 was tested in lysates of cells expressing the corresponding recombinant human

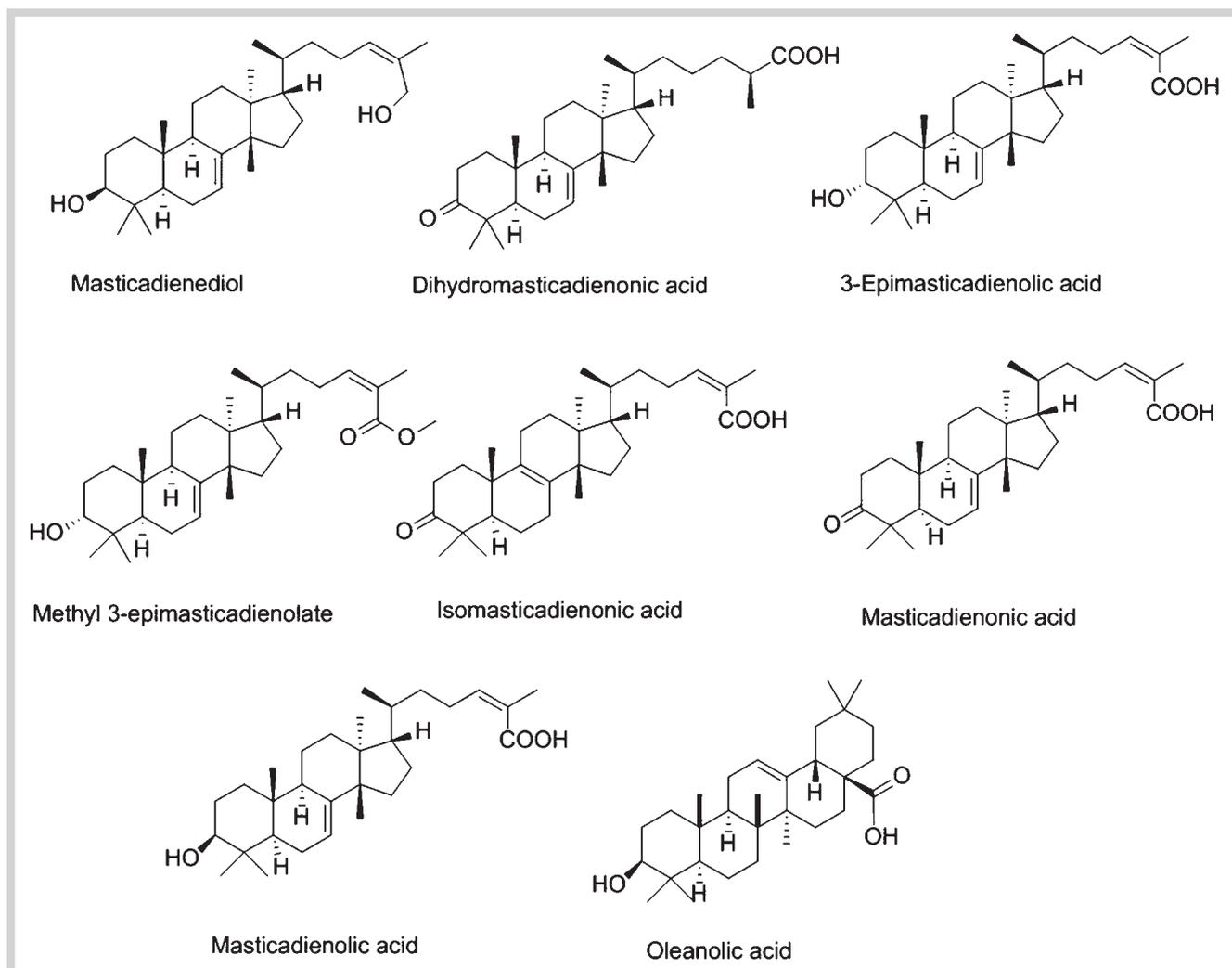


Fig. 4 *P. lentiscus* constituents found by virtual screening.

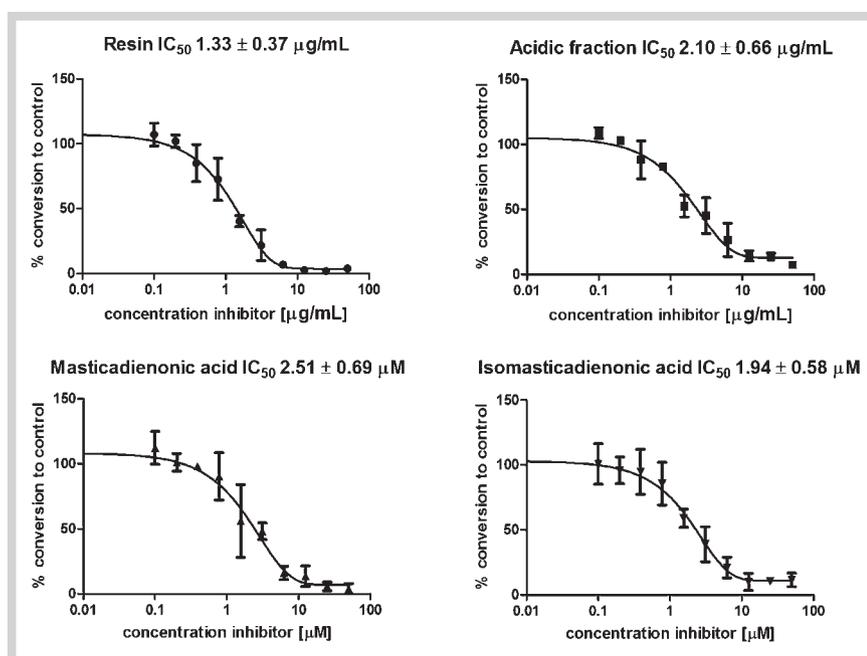


Fig. 5 Activities of *P. lentiscus* var. *chia* oleoresin, acidic fraction, masticadienonic, and isomasticadienonic acids in lysed cells expressing 11 β -hydroxysteroid dehydrogenase 1.

enzyme. All four probes, the oleoresin, the acidic fraction of the gum, masticadienonic acid, and isomasticadienonic acid dose-dependently inhibited 11β -HSD1 (● Fig. 5), but, importantly, not 11β -HSD2 (data not shown). As expected, the oleoresin that contained all the triterpenes turned out to be a potent 11β -HSD1 inhibitor with an IC_{50} value of $1.33\ \mu\text{g}/\text{mL}$, whereas the acidic fraction, containing all the acidic triterpenes, had an IC_{50} of $2.10\ \mu\text{g}/\text{mL}$. Masticadienonic acid and isomasticadienonic acid had IC_{50} values of $2.51\ \mu\text{M}$ and $1.94\ \mu\text{M}$, respectively. In contrast, the non-selective reference compound glycyrrhetic acid inhibited 11β -HSD1 and 11β -HSD2 with IC_{50} values of $0.68 \pm 0.17\ \mu\text{M}$ and $0.26 \pm 0.07\ \mu\text{M}$, respectively, in line with previously published data [28].

To evaluate how masticadienonic acid and isomasticadienonic acid bind to 11β -HSD1 and to estimate their mechanism of action, they were docked into the respective binding pocket. Both of them aligned well with each other and with the cocrystallized ligand carbenoxolone (● Fig. 6A). They did not form hydrogen bonds with the catalytic residues, but occupied the binding site, thus preventing the natural ligand from binding. Masticadienonic acid was anchored to the binding site with hydrophobic interactions and with a hydrogen bond with the backbone nitrogen of Leu217 (● Fig. 6B). Isomasticadienonic acid was proposed to bind similarly to the binding pocket, but it also formed a hydrogen bond with the cofactor NADPH (● Fig. 6C).

Discussion

In this study, a pharmacophore-based virtual screening of the natural compound database DIOS was performed. As a virtual screening filter, a previously published and refined pharmacophore model for 11β -HSD1 inhibitors was used. The model successfully recognized triterpenes, from which the majority was from *Pistacia* species. The biological evaluation of the mastic gum oleoresin and its constituents supported the hypothesis of 11β -HSD1 being one of the targets involved in the antidiabetic activity of mastic gum. To support the biological findings, binding orientations for masticadienonic acid and isomasticadienonic acid were predicted. The predicted binding modes were compared with corosolic acid (docking studies reported by Rollinger et al. [27]) and the cocrystallized ligand carbenoxolone. Isomasticadienonic and masticadienonic acids did not have the similar flipped binding orientation predicted for corosolic acid. However, their binding orientations and observed hydrogen bonds with Leu217 and the cofactor as well as the hydrophobic interactions are comparable with those of carbenoxolone. Therefore, masticadienonic and isomasticadienonic acids are suggested to act as competitive 11β -HSD1 inhibitors, like carbenoxolone.

The findings of this study show that the oleoresin of *P. lentiscus* (mastic gum), and especially masticadienonic and isomasticadienonic acids, target 11β -HSD1, which may contribute to a lowered blood glucose and improved serum fatty acids concentrations that have been observed in earlier reports on the treatment of diabetic mice with mastic gum [9]. Moreover, moronic acid, one of the triterpenes present in mastic gum [4], has been shown to exert antihyperglycemic properties in rats and to be a weak 11β -HSD1 inhibitor *in vitro* (22% enzyme inhibition at the concentration of $10\ \mu\text{M}$) [36]. Other triterpenes with a Δ^{12} -oleanene skeleton, such as oleanolic acid, which was also found as a virtual hit, and its derivatives, have been proven to inhibit 11β -HSD1 with high nanomolar IC_{50} values [37]. However, the activity of olea-

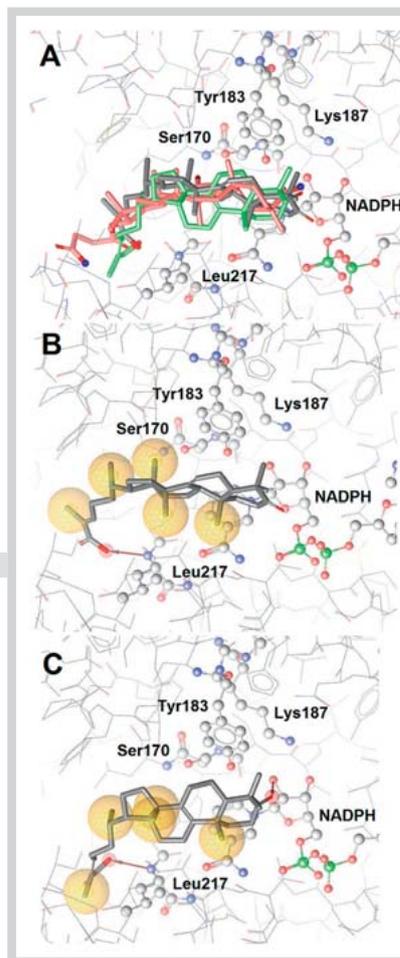


Fig. 6 Predicted binding orientations of masticadienonic and isomasticadienonic acids in the 11β -hydroxysteroid dehydrogenase 1 ligand binding site. Masticadienonic acid (red) and isomasticadienonic acid (green) occupy the same space in the binding pocket compared to the cocrystallized ligand carbenoxolone (gray) (A). Masticadienonic acid (B) was anchored to the binding pocket with hydrophobic interactions, hydrogen bonds with Leu217, whereas isomasticadienonic acid (C) forms an additional hydrogen bond with the cofactor NADPH. Hydrophobic interactions are shown as yellow spheres and hydrogen bonds as red arrows. The catalytic triad Ser-Tyr-Lys, the cofactor, and Leu217 are depicted in ball-and-stick style. (Color figure available online only.)

onic acid, a mastic gum constituent, is to the best of our knowledge unknown.

There are several studies reporting natural compounds as 11β -HSD1 inhibitors with the potential to be antidiabetic drugs. Nevertheless, most of them, like glycyrrhetic acid and curcumin, also inhibit 11β -HSD2 and may therefore not be suitable for diabetes treatment [38,39]. Other compounds like flavonone and its derivatives selectively inhibited 11β -HSD1, however, they are rather weak inhibitors [40].

Several constituents of mastic gum acting on the same or different targets could provide an explanation for the antidiabetic actions of mastic gum, such as masticadienonic, isomasticadienonic, and moronic acids inhibiting 11β -HSD1, as well as oleanonic acid activating PPAR γ . However, of these compounds, masticadienonic and isomasticadienonic acids are the most potent, experimentally confirmed 11β -HSD1 inhibiting constituents from mastic gum. In addition, at a concentration of $200\ \mu\text{M}$, oleanonic acid caused a 13-fold activation of PPAR γ , whereas masticadienonic and isomasticadienonic acids inhibited 50% of the 11β -HSD1 activity at concentrations of about $2\ \mu\text{M}$. Based on the previously performed quantification study using an GC-MS technique, these two bioactive compounds (masticadienonic and isomasticadienonic acids) account for 3.4 and 8.9% w/w in mastic gum oleoresin, respectively [4]. Thus, even at low mastic gum concentrations, the pronounced 11β -HSD1 inhibition of these two main constituents as well as probably further contributing congeners may be the main reason for the oleoresin's antidiabetic effects. However, it is currently not clear whether additional

targets are also involved which warrant further studies on the molecular mechanism of the constituents of this traditionally used herbal remedy.

In addition to the explanation for the antidiabetic effects of mastic gum, the findings of this study form an excellent basis for the discovery of new 11 β -HSD1 inhibitors from a natural origin. The pharmacophore model has proven it is able to enrich active natural compounds from a large database by identifying masticadienonic and isomasticadienonic acids as new selective 11 β -HSD1 inhibitors from natural sources.

Materials and Methods

Virtual screening

For the virtual screening, the DIOS database was composed with the Build 3D database tool of DiscoveryStudio 3.0 (2005–2010 Accelrys Software, Inc.). The database was generated with Best settings and a maximum of 255 conformations per molecule. The pharmacophore model for 11 β -HSD1 inhibition was obtained from our pharmacophore model collection. The detailed pharmacophore generation and refinement were described by Schuster et al. [32] and Vuorinen et al. [33]. Briefly, Schuster et al. developed a ligand-based pharmacophore model for 11 β -HSD1 inhibitors. The model was trained to enrich the active substances from a set of active and inactive compounds, used for virtual screening, and successfully experimentally validated. During the last years, a vast number of new 11 β -HSD1 inhibitors have been developed, and the model needed refinement in order to maintain a good predictive power. The final pharmacophore model used in this present study is a refined version of the model published by Schuster et al. The virtual screening of the DIOS database was performed with the Search 3D database tool of DiscoveryStudio 3.0 (2005–2010 Accelrys Software, Inc.) with Best settings.

Preparation of samples

P. lentiscus var. *chia* oleoresin collected traditionally (crude, large tears) was kindly provided by the Mastic Gum Growers Association (Chios, Greece). The acidic fraction (NaOH) of *P. lentiscus* var. *chia* was isolated as described in [4] after fractionation of the crude *P. lentiscus* var. *chia* oleoresin. Masticadienonic acid and isomasticadienonic acid were isolated by semipreparative HPLC-DAD (Dionex summit – preparative HPLC system) from the acidic fraction of *P. lentiscus* var. *chia* (as prepared above) as described in [41]; their purity was determined by HPLC-DAD also as described in [41]. Their structures were identified by spectroscopic (1D and 2D NMR) and analytical methods (HPLC-MS) (as shown in [41]), and additionally by comparison with published data [4]. All solvents used in this study were LC-MS grade and supplied by Merck.

Biological evaluation

Inhibition of 11 β -HSD enzyme activity was performed as described earlier [28]. HEK-293 cells stably expressing human 11 β -HSD1 or 11 β -HSD2 were harvested by trypsination, followed by centrifugation. The resulting cell pellets were frozen and stored at -80°C . For the experiments, cell pellets were suspended in TS2 buffer (100 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1 mM MgCl₂, 250 mM sucrose, 20 mM Tris-HCl, pH 7.4), sonicated, and immediately used for the activity assay. Cell lysates were incubated for 10 min at 37°C in a final volume of 22 μL containing either ve-

hicle (0.2% methanol) or the corresponding inhibitor. Glycyrrhetic acid (Sigma-Aldrich, article G10105, 97% assay purity) was used as a reference compound [28]. Inhibitors were diluted in TS2 buffer from stock solutions (10 mg/mL or 10 mM in methanol). To measure 11 β -HSD1 activity, the reaction mixture contained 200 nM [1,2-³H]cortisone and 500 μM NADPH. 11 β -HSD2 activity was measured similarly at a final concentration of 50 nM [1,2,6,7-³H] cortisol and 500 μM NAD⁺. Reactions were stopped by adding an excess of unlabeled cortisone and cortisol (2 mM each, in methanol). Steroids were separated by TLC, followed by scintillation counting and calculation of the substrate conversion compared to the methanol control. Data were obtained from three independent experiments.

Docking

Masticadienonic acid and isomasticadienonic acid were drawn with ChemBioDraw Ultra 12.0 (1986–2010 CambridgeSoft), and 3D structures were obtained using PipelinePilot (2010 Accelrys Software, Inc.). The X-ray crystal structure of 11 β -HSD1 was downloaded from the PDB (www.pdb.org [42]). For 11 β -HSD1, the PDB entry 2 BEL [43] was chosen because it is cocrystallized with carbenoxolone, a ligand that is structurally similar to the triterpenoid mastic gum compounds. The docking was performed using GOLD [44,45]. This program uses a genetic algorithm for creating low-energy binding orientations for small molecules into the binding pocket of a protein. The binding site was defined as an 8 Å sphere, centered on the hydroxyl-oxygen of Ser170 (x 3.84; y 22.49; z 13.34). ChemPLP was selected as a scoring function, and the program was allowed to terminate the docking run in case the three best-scored binding orientations were located similarly in the binding site. To ensure acceptable ligand flexibility, the program was set to flip ring corners when exploring the binding orientations. Atom types for the protein and for the ligand were defined by the program. These docking settings were validated by redocking the original ligand, carbenoxolone, for a correct reproduction of the binding mode obtained by crystallography.

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Conflict of Interest

The authors declare no conflict of interest.

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