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Research paper

The molecular mechanism of human hormone-sensitive lipase inhibition by substituted 3-phenyl-5-alkoxy-1,3,4-oxadiazol-2-ones

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ABSTRACT

Hormone-sensitive lipase (HSL) plays an important role in the mobilization of free fatty acids (FFA) from adipocytes. The inhibition of HSL may offer a pharmacological approach to reduce FFA levels in plasma and diminish peripheral insulin resistance in type 2 diabetes. In this work, the inhibition of HSL by substituted 3-phenyl-5-alkoxy-1,3,4-oxadiazol-2-ones has been studied *in vitro*. 5-methoxy-3-(3-phenoxyphenyl)-1,3,4-oxadiazol-2(3*H*)-one (compound 7600) and 5-methoxy-3-(3-methyl-4-phenylacetamidophenyl)-1,3,4-oxadiazol-2(3*H*)-one (compound 9368) were selected as the most potent HSL inhibitors. HSL is inhibited after few minutes of incubation with compound 7600, at a molar excess of 20. This inhibition is reversed in the presence of an emulsion of lipid substrate. The reactivation phenomenon is hardly observed when incubating HSL with compound 9368. The molecular mechanism underlying the reversible inhibition of HSL by compound 7600 was investigated using high performance liquid chromatography and tandem mass spectrometry. The stoichiometry of the inhibition reaction revealed that specifically one molecule of inhibitor was bound per enzyme molecule. The inhibition by compound 7600 involves a nucleophilic attack by the hydroxy group of the catalytic Ser of the enzyme on the carbon atom of the carbonyl moiety of the oxadiazolone ring of the inhibitor, leading to the formation of covalent enzyme–inhibitor intermediate. This covalent intermediate is subsequently hydrolyzed, releasing an oxadiazolone decomposition product, carbon dioxide and the active HSL form. On the basis of this study, a kinetic model is proposed to describe the inhibition of HSL by compound 7600 in the aqueous phase as well as its partial reactivation at the lipid–water interface.

1. Introduction

Insulin resistance is one parameter of the metabolic syndrome. This metabolic syndrome is often associated with high levels of free fatty acid (FFA), in addition to increased levels of triacylglycerols (TAG) and low levels of high-density lipoprotein (HDL)-cholesterol [1]. FFAs stored as TAG in adipose tissue are the main source of energy in mammals and influence various aspects of lipid and carbohydrate metabolism. Elevated plasma FFA levels impair insulin signaling, decrease glucose oxidation and glycogen synthesis [2,3] and results in decreased rates of glucose uptake. Moreover, FFA may influence both insulin sensitivity in muscle and insulin secretion in pancreas.

HSL is thought to play an important role in the mobilization of FFA from the TAG stored in adipocytes, skeletal muscle, and pancreatic β-cells (for a review, see ref. [4]). *In vivo*, HSL is activated by phosphorylation via cAMP-dependent kinase in response to various lipolytic hormones such as catecholamines. The functional significance of HSL in adipose tissue metabolism has been clarified in studies using HSL null mice [5–9]. TAG lipase activity in white adipose tissue was reduced by only 40–45% and in brown adipose tissue was similar to wild-type mice [8]. Additionally, there was a marked defect or complete absence of catecholamine-stimulated

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glycerol release in adipocytes from HSL null mice, whereas catecholamine-stimulated FFA release was still observed, but attenuated [8,9]. This apparent discrepancy in the release of glycerol and FFA from adipocytes of HSL null mice has been clarified by the observation that the basal- and catecholamine-induced diacylglycerol (DAG) content increased markedly in white and brown adipose tissue of HSL null mice [5]. Therefore, the study of HSL null mice appear to substantiate that HSL is the rate limiting-enzyme for DAG hydrolysis in adipose tissue and is essential for hormone-stimulated lipolysis. Using chiral-stationary-phase HPLC, we recently showed that HSL was found to have a pronounced stereospecificity for the *sn*-3 position of DAG [10].

Furthermore, the inactivation of HSL resulted in the loss of more than 98% of neutral cholesteryl ester hydrolase activity in both male and female null mice adrenals suggesting that HSL is critically involved in the intracellular processing and availability of cholesterol for adrenal steroidogenesis [6,7]. In line with these observations, we reported previously that HSL activity on cholesteryl esters was approximately four to five times higher than on long-chain TAG [11] and consequently, HSL could be considered more as a cholesteryl ester hydrolase than as a TAG lipase [11]. An adipose TAG lipase (ATGL) was identified [12] and proposed to be responsible for the initial step in TAG catabolism. HSL and ATGL may coordinately catabolize the TAG stored in mammalian adipose tissues [12]. Another neutral intracellular TAG hydrolase (TGH) also termed carboxylesterase 3 was shown to be associated with hepatic [13] and adipocyte lipid droplets [14,15] where it may participate in TAG turnover and fatty acid efflux. It was reported recently [16] that the ablation of TGH expression in mice ($Tgh^{-/-}$) results in decreased hydrolase activity accompanied with reduced plasma FFA and glycerol levels. $Tgh^{-/-}$ mice exhibited improved glucose tolerance and insulin sensitivity [16].

Targeting FFA metabolism may therefore offer a therapeutic approach that addresses the fundamental cause of insulin resistance and type 2 diabetes. Thus, the inhibition of HSL offers a pharmacological approach to reduce FFA levels and diminish peripheral insulin resistance. Over the last years, various HSL inhibitors, including natural products (cyclipostins from *Streptomyces* [17] and Eudesmanolides isolated from *Iva microcephala* nut [18]) and synthetic products (carbamates [19], pyrrolopyrazinediones [20] and carbazates [21]) have been described for the treatment of diabetes and lipid disorders. These reports lack however, detailed biochemical characterization of HSL inhibition by these inhibitors.

The HSL family is an increasingly large group of proteins showing structural similarities with the catalytic domain of HSL. HSL is the only carboxylester hydrolase belonging to this family of proteins having clearly detectable lipolytic activity on long-chain TAG, DAG, monoacylglycerol, cholesteryl ester and retinyl ester substrates. We showed previously [22] that compound 7600 specifically inhibits the HSL family members (HSL, esterase from the thermophilic eubacterium *Alicyclobacillus acidocaldarius* (EST2) and an esterase from hyperthermophilic archaeon *Archaeoglobus fulgidus* (AFEST)). Whereas, no significant inhibitory effects were observed with other lipolytic and non-lipolytic carboxylester hydrolases that are not members of the HSL family [22]. Surface enhanced laser desorption ionisation/time of flight (SELDI-TOF) mass spectrometry analysis of a trypsin digest of AFEST treated or not with the inhibitor showed the occurrence of an increase in the molecular masses of catalytic serine-containing peptide, which is compatible with the formation of a covalent complex with the inhibitor [22]. The aim of the present study was to investigate the inhibition properties of compound 7600 on HSL activity, as well as the molecular mechanism of this inhibition using high performance liquid chromatography and tandem mass spectrometry (LC-MS).

2. Materials and methods

2.1. Reagents

Dioleoylglycerol (diolein), tributyroylglycerol (tributyrin), cholesterol oleate, vinyl butyrate, Nonidet-P, sodium taurodeoxycholate (NaTDC), dimethylsulfoxide (DMSO) and bovine serum albumin (BSA) were purchased from Sigma–Aldrich–Fluka Chimie (St-Quentin-Fallavier, France). HPLC grade acetonitrile was obtained from Merck (Darmstadt, Germany). All other chemicals and solvents were of reagent or better quality and were obtained from local suppliers.

2.2. Substituted 3-phenyl-5-alkoxy-1,3,4-oxadiazol-2-ones

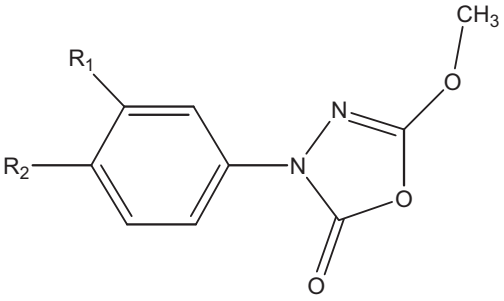
Substituted 3-phenyl-5-alkoxy-1,3,4-oxadiazol-2-ones were synthesized as described previously [23]. Compound 7600 and compound 9368 (Fig. 1) were selected as the most potent HSL inhibitors.

2.3. Proteins

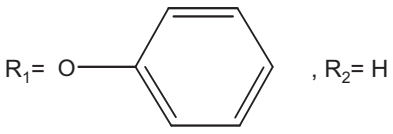
Recombinant human HSL was expressed and purified from baculovirus-infected insect cells as described previously [24]. The protein concentrations were determined using Bradford's procedure [25], with Bio-Rad Dye Reagent and BSA as the standard.

2.4. Enzyme activity measurements

Enzymatic activity was assayed by measuring the FFA released from mechanically stirred acylglycerol, using 0.1 N NaOH with a pH-stat (TTT80 radiometer, Copenhagen) adjusted to a fixed end



Compound 7600:



Compound 9368:

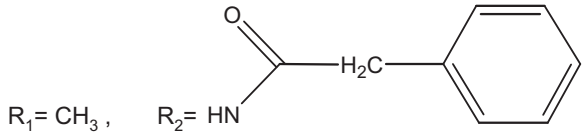


Fig. 1. Compound 7600 and compound 9368 structures.

point value. When olive oil or dioleoylglycerol were used as substrates, gum arabic was used as an emulsifier as previously described [26]. Each assay was performed in a thermostated (37 °C) vessel containing 0.25 mM Tris–HCl buffer and 150 mM NaCl. The activity of HSL was measured at pH 7.5 in the presence of BSA (2 μM, final concentration). The specific activities are expressed here in international units (IU) per milligram of enzyme. One unit corresponds to 1 μmol of FFA released per minute.

2.5. Inhibition of HSL by compound 7600 and compound 9368

The enzyme was incubated with the inhibitor, in aqueous medium for testing direct interaction between the lipase and the inhibitor in the absence of substrate [27]. Recombinant human HSL (0.2 mg ml^{-1}; final concentration 2.3 μM) was dissolved in Na_2HPO_4 buffer, pH 7.5, containing 0.1 M NaCl, 20% glycerol, 3 μM β-mercaptoethanol and 0.1% Nonidet-P. Compound 7600 or 9368 (10 mM) was pre-dissolved in DMSO for its further dispersion in the water phase. An aliquot of the above enzyme solution was incubated at 25 °C with the inhibitor at an enzyme:inhibitor molar ratio varying from 1:100 to 1:1 (final DMSO concentration ranged from 2 to 3%). The residual enzyme activity was then measured after various incubation times, using emulsions of vinyl butyrate, diolein, olive oil, cholesterol oleate or tributyrin as substrate (see above). Control experiments were performed in the absence of inhibitor and with the same concentration of DMSO. It is worth noticing that DMSO at a final concentration of less than 5% has no effect on the enzyme activity.

2.6. Treatment of the kinetic data obtained upon enzyme reactivation

In order to fit the experimental data points with the general theoretical equation derived from the model proposed by Verger et al. [28] we have used this following simplified equation:

$$P = \frac{C}{B}t + \tau\frac{C}{B}\left(e^{-t/r} - 1\right) \quad (1)$$

Where P is the product (FFA) concentration (molecule/volume) and t, time (min). $(dP/dt) = C/B$ when time tends to infinity. C and B are complex parameters resulting from a combination of various individual kinetic rate constants (for their exact definition, see reference [28]).

Under steady state conditions, C/B is the maximal reaction rate and τ (lag time) is the intercept with time axis of the asymptote of equation (1) (see Fig. 2). The values of C/B and τ are given after curve fitting of the experimental data with the equation (1), using KaleidaGraph® 3.0 software.

From equation (1), one can see that theoretically the initial rate (at $t = 0$) is equal to zero. However, in order to estimate experimentally the initial activity of HSL we measured the slope of the kinetic recordings 1 min after adding HSL-inhibitor mixture to the lipase assay.

2.7. Sample preparation for the identification by LC-MS of the oxadiazolone degradation product

Recombinant human HSL (0.2 mg ml^{-1}; final concentration 2.3 μM) was dissolved in Na_2HPO_4 buffer, pH 7.5, containing 0.1 M NaCl, 20% glycerol, 3 μM β-mercaptoethanol and 0.1% Nonidet-P. An aliquot of enzyme solution was incubated at 24 °C with compound 7600 (200 μM in DMSO). After 1 h, ethyl acetate/H_2O (200 μL) was added and the products extracted from the reaction mixture. The organic layer was separated and the solvent evaporated. The residue was taken up in ethanol (15 μL) and injected into the HPLC system. The enzymatic conversion of the compound was followed by LC-MS.

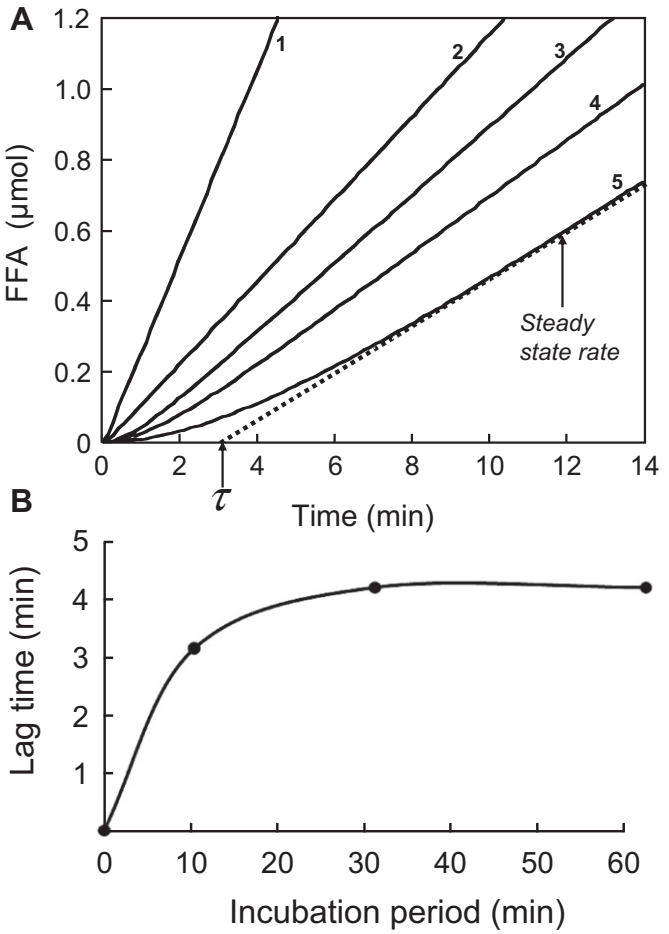


Fig. 2. (A) Typical kinetic recordings of the hydrolysis of tributyrin emulsion by HSL, using the pH-stat technique. HSL was incubated for 0 min (curve 1), 3 min (curve 2), 10 min (curve 3), 30 min (curve 4) and 60 min (curve 5) with compound 7600 at enzyme:inhibitor molar ration of 1:20. At various time intervals, an aliquot of the assay medium was injected in the pH-stat vessel, after recording the background hydrolysis for 2 min. Kinetic assays were performed in a thermostated (37 °C) vessel containing 0.5 ml tributyrin mechanically emulsified in 14.5 ml of 1 mM Tris–HCl buffer, 150 mM NaCl. Kinetic recordings are representative of three independent experiments. The dashed line corresponds to the asymptote reached under steady state conditions obtained by curve fitting the kinetics using equation (1) (see Materials and methods section). τ (lag time) is the intercept with time axis of the dashed line. (B) Variation of the lag time of HSL activity as a function of its incubation time with compound 7600, using a tributyrin emulsion. The lag times (τ) were determined as illustrated in Fig. 2 and described in Materials and methods section.

HPLC separation was performed on a Luna C18 column (100 × 4.6 mm i.d., 5 μm particle size, Phenomenex, Aschaffenburg, Germany). The HPLC system was an Agilent 1100 system (Agilent, Böblingen, Germany). HPLC was performed using the solvent systems A (acetonitrile) and B (ammonium acetate buffer, pH 2.8) at a flow rate of 1 ml/min. After equilibration of the column for 2 min with system A/system B = 50/50 (v/v), the sample was injected and the chromatography was run for 1 min with system A/system B = 50/50 (v/v). Then, over a period of 1 min the composition was changed to system A/system B = 98/2 (v/v). The chromatography was continued with this composition for 5 min. An API3000 triple-quadrupole mass spectrometer (AB/MDS Sciex, Toronto, Canada) was used for detection. A $1/x$-weighted linear model was used for the regression of peak area ratio of compound/internal standard *versus* compound concentration.

3. Results

3.1. Inhibition kinetics of HSL by compound 7600 and compound 9368

At various incubation time of HSL with compound 7600 (HSL:compound 7600 molar ratio, 1:20), aliquots were taken from the incubation medium and the residual HSL activity was measured using tributyrin emulsion as substrate. Fig. 2A shows typical kinetic recordings of the hydrolysis rates of tributyrin emulsion with HSL after various incubation time with the compound 7600. The same behavior was observed when incubating HSL with compound 9368 (data not shown). The hydrolytic activity gradually increased with time, however, reaching a steady state regime after a lag period of a few minutes. The lag time was defined as the point where the extrapolated steady-state curve intersected with the time axis (Fig. 2A). The fitting of kinetics according to equation (1) (see Materials and methods) shows a good adjustment between the experimental data and the theoretical equation (1) which allows the measurements of the initial residual activity, the steady state residual activity and the lag time. The initial residual rates decrease with increasing incubation times. The HSL activity, however, progressively increased with time and was partially restored reaching a steady state after a lag time. When aliquots of inhibited HSL were added to the lipase assay medium 10 min before adding tributyrin, similar kinetics recordings (data not shown) as in Fig. 2A were obtained. This result indicates that the reactivation process of the inhibited HSL is not the consequence of its dilution into the aqueous phase but rather to the presence of the substrate.

The reactivation phenomenon of HSL was also seen (data not shown) when using other known HSL substrates such as diolein, *p*-nitrobenzofurazan (NBD)-labeled monoacylglycerol [29] and vinyl butyrate.

The lag time values, ranging from 1 to around 4 min, were calculated from the kinetic recordings of the HSL acting on tributyrin emulsion (Fig. 2B). In the absence of inhibitor compound 7600, the kinetic was linear and no lag time was observed. However, on the presence of the inhibitor the lag time increases as function as the incubation period. When the activity is assayed just after mixing the enzyme with the inhibitor ($t = 0$ min), the low value of the lag time is comparable to those found in the absence of compound 7600 and could be attributed to the mixing time required for the injected enzyme sample to be homogeneously distributed. When the incubation period increases (10, 30 and 60 min), the values of the lag time were higher (a plateau value of around 4 min) (Fig. 2B).

Fig. 3A shows the residual HSL activity measured under steady state conditions as function of the incubation time of the enzyme with 20 molar excess of compound 7600. After few seconds of incubation, approximately 50% of the residual HSL activity was measured on tributyrin (Fig. 3A) or vinyl butyrate (data not shown) emulsions. This HSL residual activity further decreases and reaches a plateau value at approximately 35% after 60 min of incubation. Control experiments without inhibitor were performed in parallel to each assay and showed linear kinetics without significant change in HSL activity. The effects of increasing concentrations of compound 7600 on the residual steady state HSL activity is shown in Fig. 3C. The concentration of compound 7600 which reduced the HSL activity to 50% (IC_{50}) was found to be around 250 nM.

Fig. 3B shows the residual HSL activity measured under steady state conditions as function of the incubation time of HSL with 20 molar excess of compound 9368. This HSL residual steady state activity further decreases rapidly and reached approximately 25% when incubating the HSL and the compound 9368 for a few seconds and reached a plateau value corresponding approximately to 90% of inhibition. Fig. 3D shows the effects of increasing concentrations of compound 9368 on the residual steady state HSL activity. The IC_{50} of this inhibitor was found to be around 90 nM.

3.2. Products identification of the HSL-catalyzed hydrolysis of compound 7600

The possibility that HSL inhibitors investigated in this study are substrates of HSL was checked for compound 7600. At various

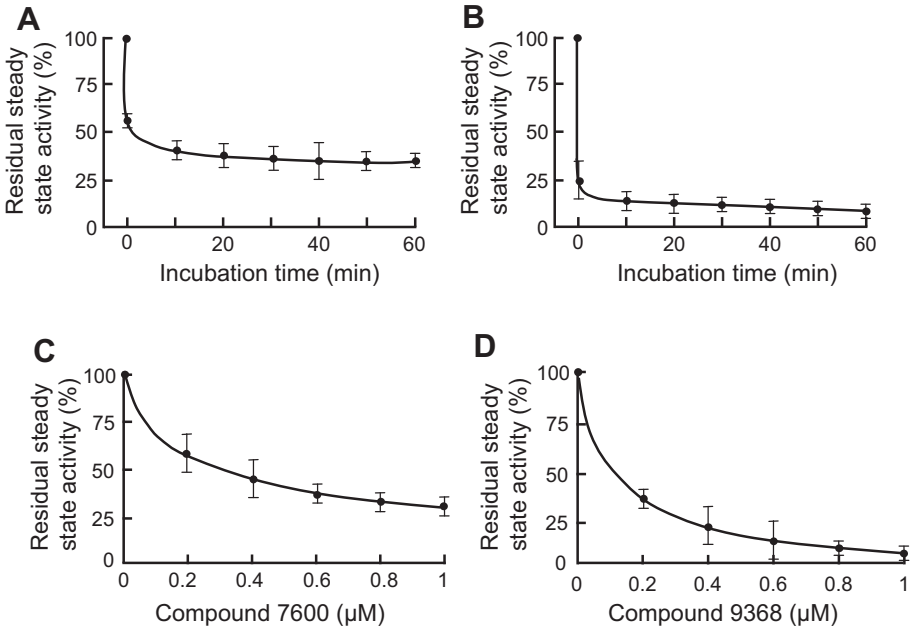


Fig. 3. HSL residual activity, measured using the pH-stat technique, under the steady state conditions using a tributyrin emulsion as substrate. (A) and (B), HSL steady state residual activity measured as function of the incubation time with compound 7600 and compound 9368, respectively, with 20 molar excess of inhibitor. (C) and (D), HSL steady state residual activity measured as function of compound 7600 and compound 9368 concentrations, respectively, after an incubation period of 60 min. The values of the steady state residual activity of HSL were determined as described in Materials and methods section. The values are expressed as means ± S.D. ($n \geq 3$ for each activity assay).

incubation time of HSL with 20 molar excess of compound 7600, aliquots were taken from the incubation medium and the resulting reaction products were analyzed by LC-MS as described in Materials and methods. As shown in Fig. 4A, liquid chromatography analysis of HSL-catalyzed hydrolysis of compound 7600 revealed three peaks having a retention time of 1.63, 1.70 and 1.90 min, respectively. A control experiment without HSL was performed (Fig. 4B) and revealed two peaks having a retention time of 1.70 and 1.90 min, respectively. The UV peak (RT 1.70 min) is probably due to an impurity within the buffer system. Mass spectra were recorded for the peaks at 1.90 and 1.63 respectively. The mass-spectrum at 1.90 shows the mass (M + H 285) corresponding to compound 7600 (Fig. 4B, insert), whereas the main peak (M + H 259) corresponds to the product of hydrolysis (Fig. 4A, insert). According to the proposed mechanism of hydrolysis the mass corresponds to methyl 2-(3-phenoxyphenyl)hydrazinecarboxylate (compound P). This metabolite has been resynthesized and co-elutes with the metabolite (RT 1.63, data not shown).

In parallel, the HSL activity was measured using the pH-stat technique. The kinetic of compound 7600 disappearance and the resulting product appearance are shown in Fig. 5. We notice a progressive enzymatic conversion of the inhibitor with a progressive increase of the reaction product (compound P). Almost complete conversion of the compound 7600 to compound P was achieved within 30 min incubation of the enzyme with the inhibitor (Fig. 5).

4. Discussion

We showed that compound 7600 and compound 9368 from the oxadiazolone family are potent inhibitors of the recombinant human HSL ([22] and this study). This inhibition occurs within

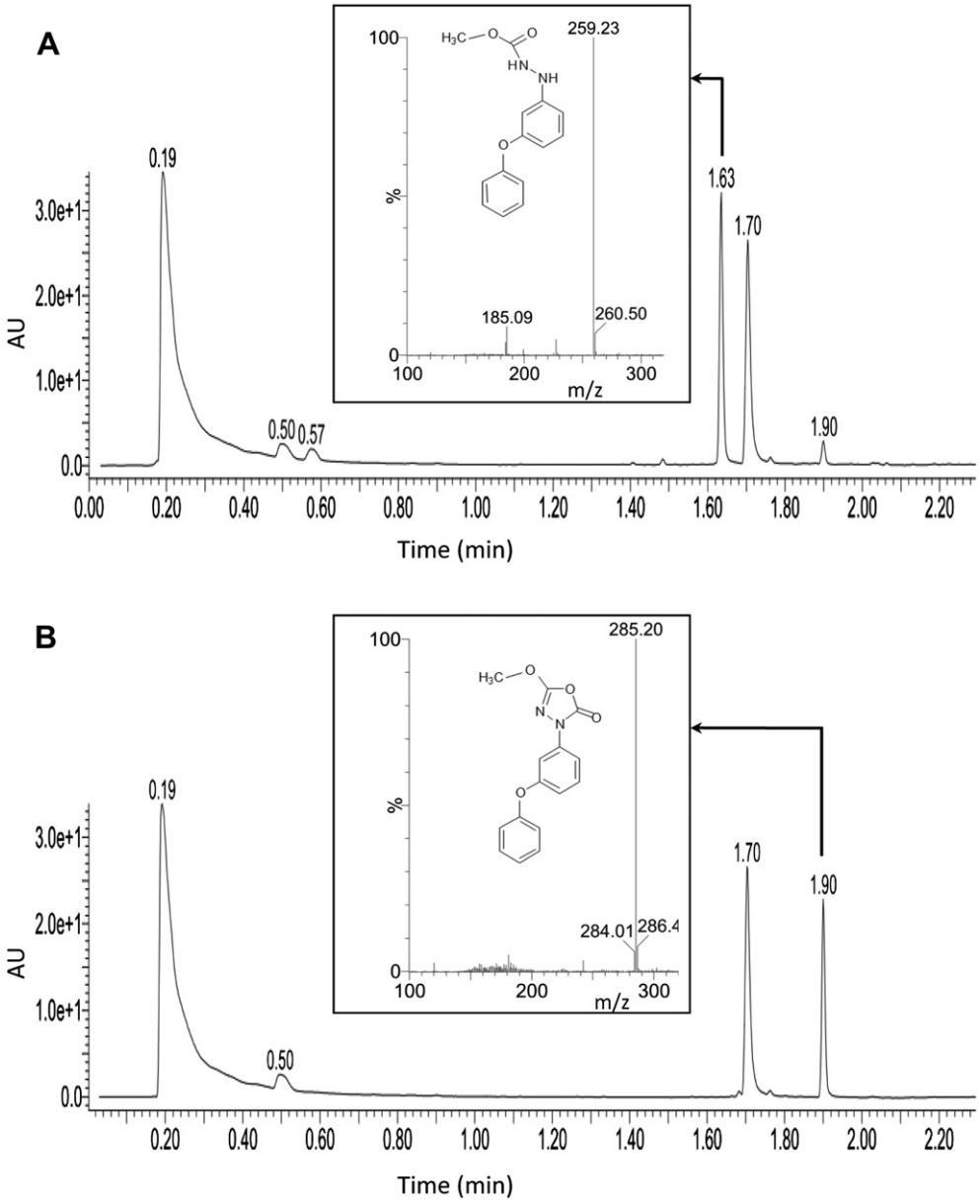


Fig. 4. Identification by LC-MS of the product generated after the hydrolysis of the compound 7600 by HSL. HSL was incubated with compound 7600 (200 μM in DMSO). An aliquot of enzyme/compound 7600 was analyzed by LC-MS (A). Control experiment of compound 7600 without HSL is shown in (B). The mass-spectrum at 1.63 (A, insert) shows the mass (M + H 259.23) corresponding to the product of hydrolysis (compound P), whereas the mass-spectrum at 1.90 (B, insert) shows the mass (M + H 285.20) corresponding to compound 7600.

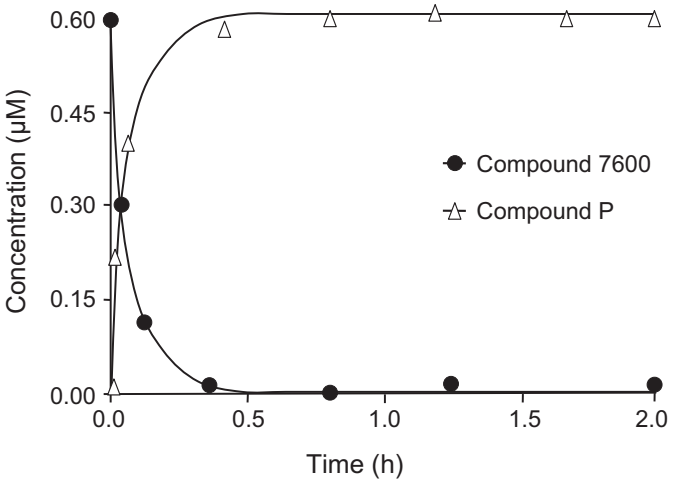


Fig. 5. Kinetic analysis by LC-MS of HSL-catalyzed compound 7600 disappearance. An aliquot of enzyme solution was incubated at 24 °C with compound 7600 at an enzyme:inhibitor molar ratio of 1:1 for 1–24 h. HPLC separation was performed in Materials and methods section.

a few minutes both in the absence and presence of detergent such as bile salts in the incubation medium. This result is in line with the fact that the catalytic serine of HSL is highly reactive and readily accessible on the contrary to what is observed in many lipases [24]. From the 3D modeling of the HSL and the distribution of the hydrophobic surfaces (see Fig. 6), HSL share same similarity with *Fusarium solani pisi* cutinase [30], a lipase that lacks the peptide loop (lid) which control the substrate access to the active site.

Compound 7600 efficiently inhibits other HSL family members, such as EST2 from the thermophilic eubacterium *A. acidocaldarius* and AFEST from the hyperthermophilic archaeon *A. fulgidus*. In contrast, carboxylester hydrolases that are not belonging to the HSL family were or poorly inhibited by compound 7600 under the same experimental conditions [22]. The inhibition of carboxylester hydrolases belonging to the HSL family resulted in a covalent modification of the active site serine and in a concomitant increase in the molecular mass of the protein, corresponding in size to the expected molecular mass of the inhibitor [22].

When HSL was incubated with compound 7600 and its residual activity was tested as function of time, two phenomena were observed: first the initial rate of substrate hydrolysis was reduced with time, second a partial enzyme reactivation was observed

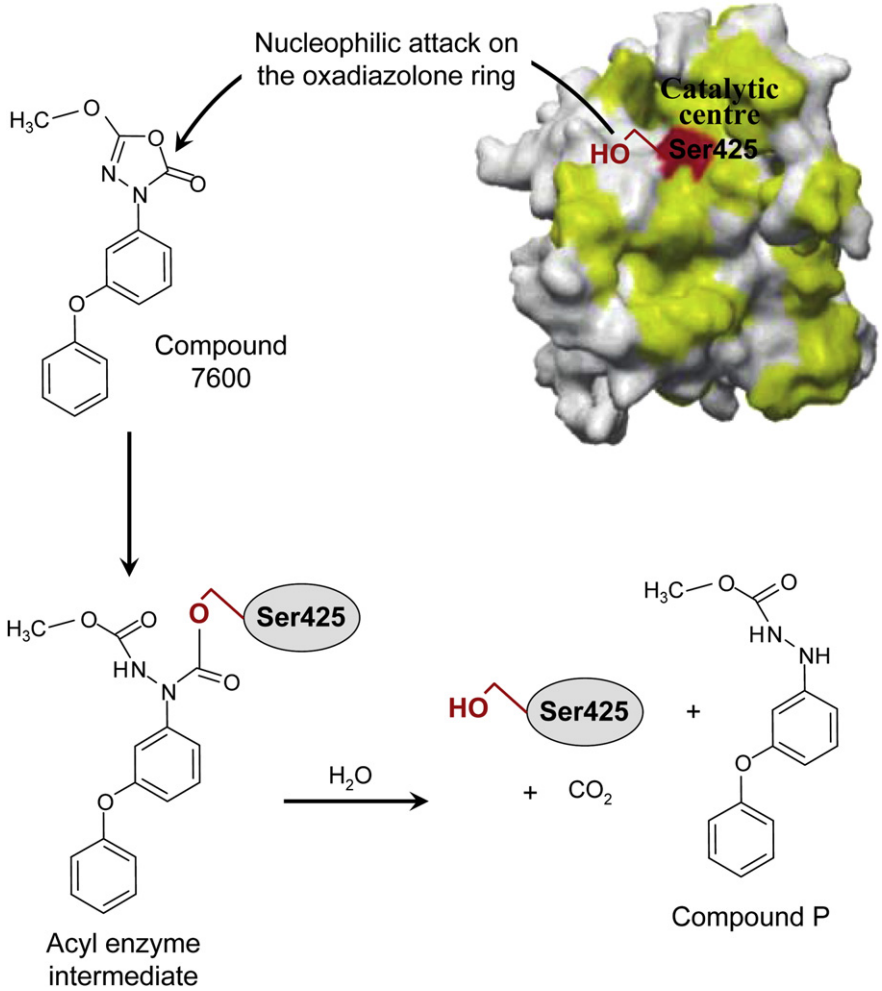


Fig. 6. The mechanism possibly underlying the inhibition exerted on recombinant human HSL by compound 7600. The acyl enzyme is formed by the nucleophilic attack by the hydroxy group of the enzyme's catalytic Ser425 on the carbonyl atom of the carbonyl moiety of the inhibitor's oxadiazolone ring, leading to the formation of an enzyme-bound intermediate. In the presence of H_2O molecule, this intermediate will be hydrolyzed to liberate active HSL and compound P. In the model of the catalytic domain of HSL, Surface distribution of hydrophobic side chain residues (V, A, L, I, W, and F), indicated in yellow, the active site serine is indicated in red [24]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

after a lag phase. These finding were obtained with tributyrin (see Fig. 2), diolein, vinyl butyrate and cholesterol oleate as substrates (data not shown). The activity of HSL toward cholesterol esters is 4- to 5-fold higher than on long-chain TAGs. However, we used in this study a partly soluble TAG such as tributyrin. We showed previously (see ref. [11]) that the HSL specific activity on emulsified tributyrin is comparable to the one on cholesterol oleate (18 ± 2 μmol min^{-1} mg^{-1}). Furthermore, from technical point of view, the emulsified tributyrin substrate for lipases is very easy to use within the pH-stat technique. This reactivation phenomenon indicates that compound 7600 is a transient inhibitor (pseudo-substrate) of HSL in which the enzyme acylation step is followed by a rate limiting deacylation reaction step of a fraction of the covalent HSL–compound 7600 complex during the lipase assay. The complete recovery of the activity of the inhibited HSL was never observed. Similar observations have been previously reported for the inhibition of human pancreatic lipase [31,32] and human carboxylester lipase [33] by tetrahydrolistatin, a potent inhibitor of carboxylester hydrolases. The complete recovery of the original activity of the enzyme was partially (human pancreatic lipase) or almost completely (human carboxylester lipase) restored after 24 h of incubation with tetrahydrolistatin [32,33]. Furthermore, it has been reported that the inhibition of bovine lipoprotein lipase [34] or *Rhizopus oryzae* lipase [35] by tetrahydrolistatin were found to be reversible in the presence of a lipid–water interface. It has been suggested that a stoichiometric enzyme–inhibitor covalent complex of a long-lived acyl-enzyme type is formed between the open β-lactone ring of tetrahydrolistatin and the catalytic serine of pancreatic lipase [31,32,36,37]. This complex was then slowly hydrolyzed, leaving an intact enzyme and the β-lactone ring of tetrahydrolistatin in its open (inactive) form.

In our work, the reversible mechanism of HSL inhibition by compound 7600 was further investigated by analyzing the enzymatic hydrolysis of the inhibitor by HSL using LC-MS. The results show clearly that compound 7600 is a pseudo-substrate of HSL as attested by the production of compound P (see Figs. 4–6). The mechanism could involve a nucleophilic attack by the hydroxy group of the catalytic Ser of the enzyme on the carbon atom of the carbonyl moiety of the oxadiazolone ring of the inhibitor. This attack leads to the formation of a stoichiometric enzyme–inhibitor covalent complex of a "long-lived" acyl-enzyme type is formed between the open oxadiazolone ring of compound 7600 and the catalytic serine of the lipase leading to of covalent enzyme–inhibitor intermediate. The last step is the deacylation reaction in which the compound P is produced (see Fig. 6).

In light of the above results, one can reasonably hypothesized that the covalent binding of compound 7600 to HSL happens in two conformations as depicted in the lower panel of the kinetic model shown in Fig. 7. A first fraction of the lipase molecules will form in the aqueous phase a covalent complex with the open oxadiazolone ring of compound 7600 in a first conformation (E–compound $7600o_1$) whereas the other fraction will also form a covalent complex with compound 7600 but in a second conformation (E–compound $7600o_2$) (Fig. 7). The formation of a non-covalent HSL–compound 7600c (Compound 7600 in its closed conformation) is not expected to occur, since results using MALDI-TOF mass spectrometry have shown the presence of only one form of HSL–compound 7600 complex [22]. Indeed, if the non-covalent HSL–compound 7600c complex exists, mass spectra should contain, in addition to the covalent HSL–compound 7600o complex, the spectra of pure enzyme [22].

In the assay system, when the mixture (E, compound 7600c, E–compound $7600o_1$ and E–compound $7600o_2$) is added to a TAG emulsion, the less stable complex (E*–compound $7600o_1$) (* indicates molecules at the water/lipid interface) dissociates by an interesterification mechanism involving TAG and characterized by a k_{T1} rate constant. Thus the reaction rate increases, due to the progressive release of an unstable acyl-HSL complex (E*–FFA). This progressive reactivation process is characterized by the existence of lag times (see Fig. 2). The fact that the reactivation of HSL does not reach 100% at the end of the kinetics could be explained by the existence of the second fraction of the enzyme which is more strongly inhibited by compound 7600 (E*–compound $7600o_2$) and which remains stable, at least during the recording kinetic period. In other words this means that k_{T1} is much higher than k_{T2}. The co-existence of two different forms of compound 7600 bound to HSL could be due to two different orientations of this inhibitor molecule in the catalytic cavity of the lipase molecule. The observed reactivation phenomenon could be due to the existence of a large proportion of E–compound 7600o which can dissociate by an interesterification mechanism involving TAG and a negligible proportion of E–compound 7600o which remain stable during the recording kinetic period.

It is worth noticing that the reactivation phenomenon is hardly observed (data not shown) when incubating HSL with compound 9368. These results probably indicate that compound 9368 is a potent inhibitor of HSL in which the enzyme forms a stable, covalent and long-lived acyl-enzyme complex. One can hypothesized that the covalent binding of compound 9368 to HSL happens also in two open conformations: A first fraction of the lipase molecules will form in the aqueous phase a covalent complex with the open oxadiazolone ring of compound 9368 in a first conformation (E–compound $9368o_1$) whereas the second fraction will form a covalent complex with compound 9368 in a second conformation (E–compound $9368o_2$). The fact that the reactivation of HSL was difficult to observe with compound 9368 could be explained by the existence of a negligible proportion of the E–compound 9368o, which can dissociates by an interesterification mechanism involving TAG and the existence of a large proportion of the E–compound 9368o which is strongly inhibited by compound 9368 and which remains stable during our experimental recording kinetic period.

Various examples were reported in line with the existence of two configurations of the ligand molecules in the active site serine of the lipase. Cygler et al. [38] have reported the three dimensional (3D) structures of covalent complexes of *Candida rugosa* lipase with two enantiomeric (1*R* and 1*S*-menthyl hexylphosphonate) transition-state analogs for the hydrolysis of menthyl esters. The 1*R* enantiomer, derived from the fast-reacting enantiomer of menthol, and the 1*S*, derived from the slow-reacting enantiomer, were found to bind to the lipase in two different orientations. Furthermore, in contrast to the fast-reacting enantiomer, in the lipase/slow-reacting enantiomer the imidazole ring of the residue His-449 was found to have rotated, thus disrupting its hydrogen bond with the menthol oxygen atom, which probably explains the differences in reactivity between the two enantiomers [38]. Furthermore, Egloff et al. [39] have reported that the two enantiomers of a C11 alkyl phosphonate compound, covalently bound to the active serine residue of HPL, occupy two different conformations with a different occupancy levels (57% and 43% for *R* and *S* conformations, respectively). As in the case of *C. rugosa* lipase reported above, in one conformation the methoxy oxygen of the first enantiomer forms a hydrogen bond with the residue His-263, whereas in the second enantiomer this hydrogen bond is lacking [39].

Uppenberg et al. [40] performed structural studies on the lipase B from *Candida antarctica*, which showed the presence of a stereospecific pocket for secondary alcohols. In their conclusion to this study, the authors stated that they could not rule out the possibility that the two enantiomers of the phosphonate inhibitor may bind competitively to the serine. This would give rise to a mixture of

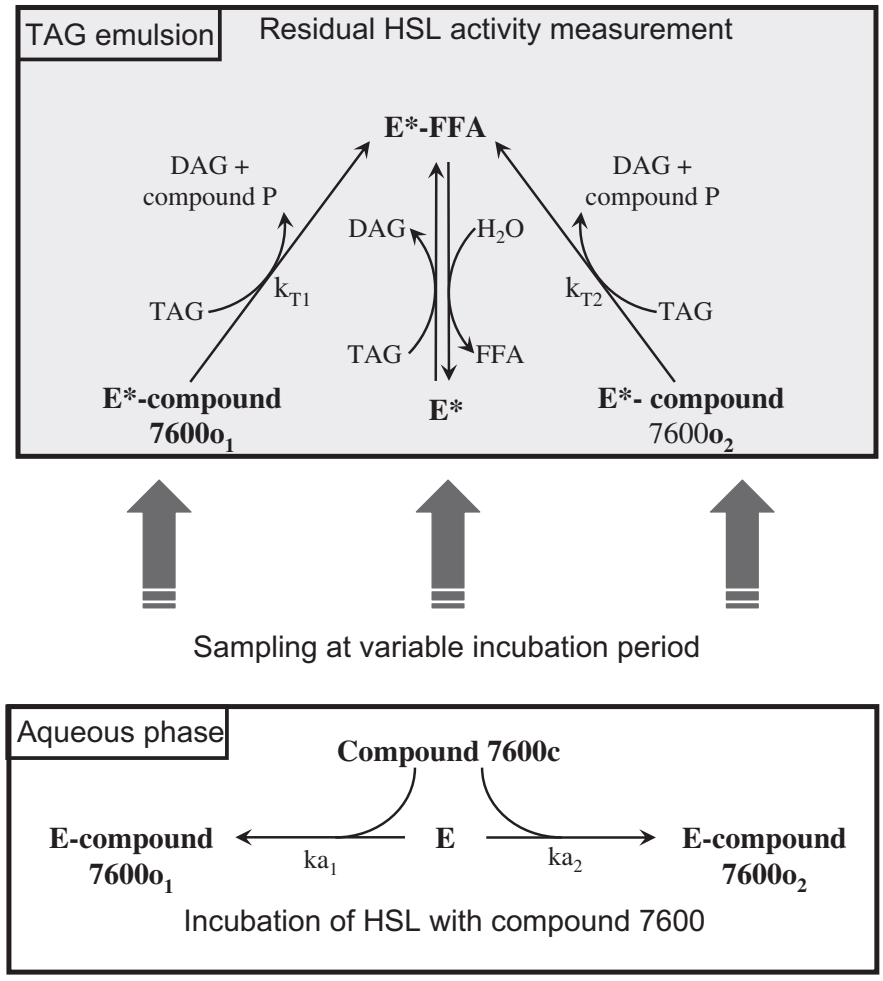


Fig. 7. Kinetic model illustrating the inhibition of HSL by compound 7600 in the aqueous phase and its reactivation at a lipid–water interface. Symbols and abbreviations are as follows: E, free enzyme (molecule/volume); E*, interfacial enzyme (molecule/surface); E*–FFA, interfacial enzyme–fatty acid complex (molecule/surface); compound 7600c, the closed and reactive compound 7600 in the bulk (molecule/volume); E–compound $7600o_1$, form 1 of the covalent enzyme–compound 7600 complex in the bulk (molecule/volume); E–compound $7600o_2$, form 2 of the covalent enzyme–compound 7600 complex in the bulk (molecule/volume); E*–compound $7600o_1$, interfacial form 1 of the covalent enzyme–compound 7600 complex (molecule/surface); E*–compound $7600o_2$, interfacial form 2 of the covalent enzyme–compound 7600 complex (molecule/surface). TAG, triacylglycerol at the interface (molecule/surface); DAG, diacylglycerol at the interface (molecule/surface).

conformations in the acyl and alcohol moieties of the inhibitor present in the active site pocket. The enzyme's preference for one of the two enantiomers is therefore mainly governed by the size of the two side chains and their ability to make favorable interactions in the active site of the enzyme. Yapoudjian et al. [41] have resolved the 3D-structure of an inactive lipase mutant from *Thermomyces lanuginosus* lipase co-crystalised with the oleic acid in mixed micelles with bile salts. In this structure, the oleic acid was found to bind to the catalytic cavity of the lipase in two different orientations. In a lipase molecule, oleic acid occupied a conventional *sn-1* binding site, and in a second lipase molecule, oleic acid was trapped in an unexpected fashion, being rotated approximately 180° in respect to the main *sn-1* lipase binding site alkyl chain binding site.

In view of the structural studies mentioned above on the various ligand configurations present at the active sites of lipases, the resolution of the 3D-structure of oxadiazolone molecules bound to HSL would provide direct support for the validity of the kinetic model presented in Fig. 7.

Targeting FFA metabolism may offer a therapeutic approach that address the fundamental cause of insulin resistance and type 2 diabetes and may thus lead to the development of new therapeutic methods. The data obtained in this study on the specific inhibition of HSL should therefore be of pharmacological interest and could be used to find means of reducing FFA levels and decreasing peripheral insulin resistance.

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