

Selection of orlistat as a potential inhibitor for lipase from *Candida* species

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Abstract:

Infections caused by *Candida* species manifest in a number of diseases, including candidemia, vulvovaginal candidiasis, endocarditis, and peritonitis. *Candida* species have been reported to possess lipolytic activity due to the secretion of lipolytic enzymes such as esterases, lipases and phospholipases. Extra-cellular hydrolytic enzymes seem to play an important role in *Candida* overgrowth. Candidiasis is commonly treated with antimycotics such as clotrimazole and nystatin. The antimycotics bind to a major component of the fungal cell membrane (ergosterol), forming pores that lead to death of the fungus. However, the secondary effects caused during such treatment have aroused a need to develop a treatment based on lipase inhibition. Nonetheless, no such lipase inhibitors for candidiasis treatment are currently available. Thus, we have performed a docking study with the natural inhibitor, orlistat or tetrahydrolipstatin. Our results have shown ten possible binding inhibitors to *Candida rugosa* lipase (CRL), out of which one possibility was selected, based on the weakest inter-atomic distance of 2.7 Å. Therefore, we propose the selection and design of a potential inhibitor candidate, orlistat for the treatment of candidiasis infections. However, this study has to be supported with *in vitro* and *in vivo* experiments to demonstrate the effectiveness of orlistat in lipase inhibition.

Keywords: tetrahydrolipstatin, CRL, Hyperchem, GOLD, Genetic algorithm.

Background:

Candidiasis is the most common oral fungal infection caused by candida species in man and manifests in a variety of clinical guises ranging from pseudo-membranous (thrush), erythematous, and hyperplastic variants to more recently described linear gingival erythema associated with HIV infection [1]. *Candida* is ubiquitous and more than 200 species have been described. Some species are part of our microbiological flora and only 10% are known to be responsible for infections in people [2]. The ARTEMIS Global Antifungal Surveillance Program showed that *C. albicans* was the most common (63–70%) candidal cause of invasive fungal infections, followed by *C. glabrata* (44%), *Candida tropicalis* (6%), and *Candida parapsilosis* (5%) [3]. *Candida rugosa* has been rarely reported as a human pathogen. Recently, Colombo *et al.*, (2003) have evaluated a cluster of *Candida rugosa* candidemia cases occurring in six

hospitalized patients from a tertiary care teaching hospital in Sao Paulo, Brazil [4]. Polyene antibiotics nystatin (NYS) and amphotericin B (AmB) have been incessantly used in the treatment of topical (NYS) and systemic (AmB) fungal infections for more than 50 years now. The advantage of administering these compounds, which are more efficient and not replaceable with other agents belonging to different families of antifungal compounds, e.g. azoles, is their wide spectrum of activity towards pathogenic fungi and yeasts. However, their application is accompanied by serious side effects, resulting from compositional similarity between host and fungi cells [5]. Due to these secondary effects, other treatments based on the hydrolytic activity of extracellular secreted enzymes of fungi cells are needed [6]. An increasing amount of evidence associates lipases with microbial virulence. Putative roles of microbial extra-cellular lipases include the digestion of lipids for nutrient

acquisition, adhesion to host cells and host tissues, synergistic interactions with other enzymes, unspecific hydrolysis due to additional phospholipolytic activities, initiation of inflammatory processes by affecting immune cells and self-defense by lysing the competing microflora. The importance of extracellular secreted lipases has been demonstrated in *C. albicans* and *C. parapsilosis*. Additionally, Trofa *et al.*, (2009) have shown that disruption of lipases attenuates damage associated with *C. albicans* and *C. parapsilosis* murine infections. Hence, lipase has been identified as a possible target for the development of novel anti-fungal therapeutic compounds [7]. Orlistat or tetrahydrolipstatin is a competitive inhibitor of pancreatic lipase (PL) with β lactone cycle incorporated into a carbon skeleton. This molecule is an irreversible inhibitor of human pancreatic lipase with an IC₅₀ value of 0.14 mM. The inhibitory activity of this molecule is lost when the β lactone ring is opened. It is currently used as approved anti-obesity drug [8]. Although it is one of the best-selling drugs worldwide, it has certain unpleasant gastrointestinal side effects like oily stools, oily spotting, and flatulence among others [8].

Lipases belong to the family of carboxylic ester hydrolases, also known as tri-acylglycerol hydrolases (EC.3.1.1.3). The physiological role of lipases is to hydrolyze triglycerides into diglycerides, monoglycerides, fatty acids and glycerol. Lipases also have the ability to perform synthetic reactions such as esterification (reaction between acid and alcohol), trans-esterification (ester and alcohol) and the inter-esterification (ester and ester). Lipases have catalytic properties that vary according to species. The mechanism of lipase enzyme shows some similarities with the active serine proteases. Unlike other hydrolases, the active site of lipases is usually covered with a peptide loop formed by an amphiphilic α helix of about fifteen amino acids, that acts as flap (lid) [9-11]. When α helix covers the active site, the enzyme is in its closed or inactive conformation. In this conformation, the hydrophobic face of the amphiphilic helix interacts with hydrophobic residues surrounding the active site while its hydrophilic face interacts with water molecules. The substrate cannot be interacting with the catalytic triad. In the open or active conformation of the enzyme which is a result of interfacial activation mechanism, there is a shift of α helix constituting the cover. The hydrophobic face of the helix facing inward before the active site exposed to the solvent, creating a hydrophobic surface, assumed to interact with the interface water / fat. The active site of the enzyme is then accessible to the substrate.

Based on the crystallographic data, some residues, different from the catalytic triad appear to be important in the catalytic mechanism. These residues form what is called the oxyanion hole. Their role is mainly to stabilize reaction intermediates, such as the tetrahedral intermediates. In the case of lipase from *Candida rugosa*, it is the nitrogen amide of Gly123 and Gly124 and Ala210 that appears to perform the same function [12]. The hydrolysis of a carboxylic ester by the catalytic triad can be divided into six main stages. First, the carbon of the carboxylic function of the substrate undergoes nucleophile attack of the hydroxyl group of

serine whose nucleophilicity is enhanced by the histidine residue following the formation of a hydrogen bond. The imidazole ring of histidine becomes protonated and positively charged. This positive charge is stabilized by a charge of an acidic residue (Asp or Glu) (Figure 1(1)). This results in the formation of the first tetrahedral intermediate, stabilized by two hydrogen bonds with residues of the oxyanion hole (Figure 1(2)). Subsequently, there is release of a molecule of alcohol, formation of the acyl-enzyme (Figure 1(3)) and nucleophile attack of the acyl-enzyme by a water molecule (Figure 1(4)). This second nucleophile attack results in the formation of a second tetrahedral intermediate, stabilized by the oxyanion hole (Figure 1(5)). Finally, there is the fatty acid release and return of the enzyme in its original conformation (Figure 1(6)) [12-14]. Hence, we have studied the use of orlistat as another candidiasis treatment with low secondary effects.

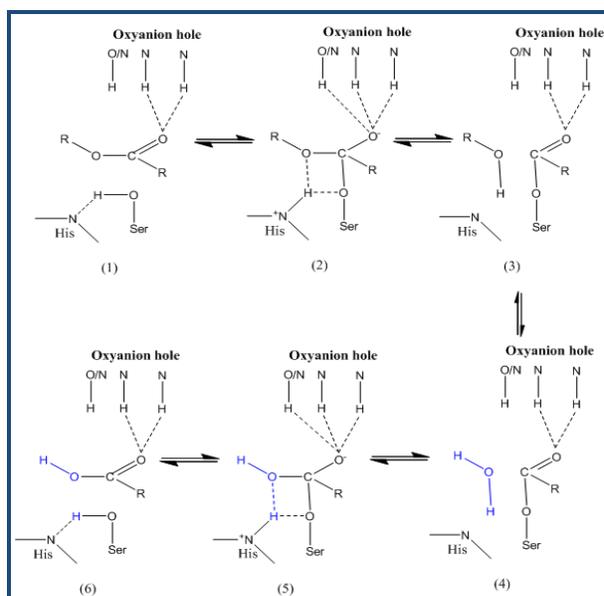


Figure 1: Schematic representation of the lipase ester-cleavage reaction pathway: (1) non-covalent Michaelis complex; (2) first tetrahedral transition state; (3) formation of the acyl-enzyme and release of an alcohol molecule; (4) attack of a water molecule on the acyl-enzyme; (5) second tetrahedral transition state; (6) release of the fatty acid [12].

Methodology:

Building the 3D structure of orlistat:

The inhibitor model was built using Hyperchem software (www.hyper.com) based on Lewis structure. Atoms have been chosen from dialog box (default elements) in build menu. The 3D structure of the orlistat is shown in Figure 2.

Optimizing the Structure of inhibitor model:

In this step, the inhibitor model structure was minimized by performing a molecular mechanics optimization using MM+ force field and Polack Ribier algorithm to obtain the most stable structure geometry (Figure 1).

Energetic calculations:

A single point calculation was performed in order to compute the energy and gradient of the inhibitor model, this

method allowed the calculation of the total energy and gradient before the geometry optimization.

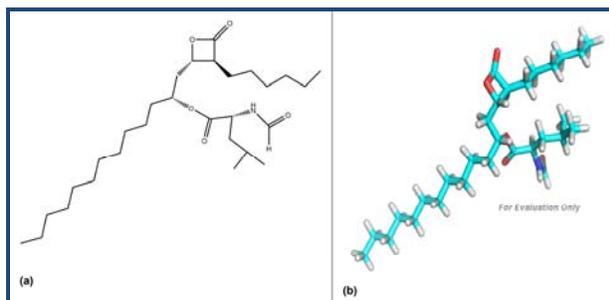


Figure 2: The 2D (a) and 3D (b) structure of orlistat.

Enzyme structure optimization:

The crystal structure coordinates of the lipases enzyme (1CRL for *Candida rugosa* lipase and PDB_ID: 1LPA for pancreatic human lipase) was obtained from the Protein Data Bank (PDB) (<http://www.rcsb.org>). Water molecules, hetero atoms and ligands such as sugars were removed [15]. All hydrogen atoms were added to the protein including those necessary to define the correct ionization and tautomeric states of amino acid residues using Hyperchem software. A two-step procedure was set up for the energy minimization of protein using the same software. In the first step, all hydrogen atoms in the protein were allowed to optimize. The hydrogen locations are not specified by the X-ray structure but these are necessary to improve the hydrogen bond geometries. In the second stage, all protein atoms were allowed to relax. Minimization in both stages was performed using 100 steps of steepest descent and 2000 steps of conjugate gradient algorithm [16].

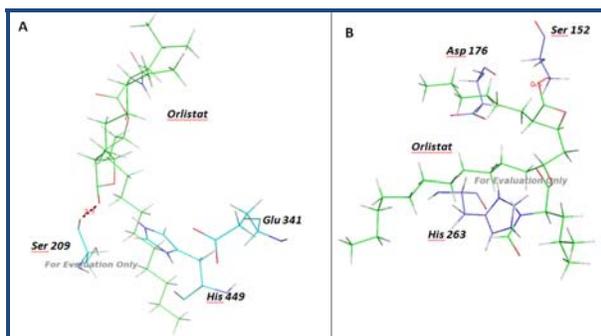


Figure 3: The binding modes of orlistat (shown as green lines) in the active site of both inter-atomic distances (shown as red dashes) represented in A: CRL, B: HPL, (2.7Å; 0.5 Å respectively). Triad catalytic shown with blue lines for both lipases.

Docking procedure of orlistat:

Docking of the inhibitor in the active site of the two lipases was carried out using GOLD 4.1.2 software (Genetic optimisation of ligand docking). The procedure consisted of three main parts: (1) A scoring function to rank different binding modes; the Gold score function is a molecular mechanics-like function with four terms, was used. (2) A mechanism for placing the ligand in the binding site; GOLD uses a unique method to do this, which is based on fitting

point. (3) A search algorithm to explore possible binding modes; GOLD uses a genetic algorithm (GA) [17-18]. This method allows a partial flexibility of protein and full flexibility of ligand [19] for each of the 10 independent GA runs.

Discussion:

Prior to the docking, amino acids and the anatomy of the active site of both lipases (CRL and HPL) have been studied by consulting literature of previous works and also by using the software PyMOL to understand the specificity of the active site towards the substrate. The funnel-like binding pocket of HPL (13*4.5 Å at its base) consists of two hand walls. Its left-hand wall is the lowest of all lipase binding sites (4.5 Å). In addition, HPL is the only lipase where this left-hand wall is lower than both the left and the right-hand walls in the front view [20]. Similarly, the lid or the amphiphilic component of Cys 237 to Cys 261 [11] forms the left wall to the binding site of alcohol. The β 5-loop lies opposite to the lid and constitutes the right-hand wall of the alcohol binding site [20]. The binding pocket of CRL is exceptional and completely differs from those of the other lipases. Here, the scissile fatty acid binding site is located in a tunnel inside the protein with a wide entrance at the right-hand side. This tunnel is at least 22 Å long with a diameter of about 4 Å. In the free CRL, this tunnel can hardly be predicted, since it is blocked by side chains. It is formed by G124 and A210 (oxyanion hole), F125, the catalytic S209, M213, V245, P246, F296, S301, L302, R303, L304, L307, F345, Y361, F362, S365, F366, V409, L410, L413, G414, F415, F532 and V534 [20].

The results of docking have shown 10 binding possibilities of orlistat in the active site of both lipases, and we have accepted one of them according to the weakest inter-atomic distance (ID) between the oxygen atom of the hydroxyl group of the catalytic amino acid Ser (for CRL: Ser209; for HPL: Ser152) and the ketone function of β lactone cycle of orlistat. It is attempted that this distance allows the formation of covalent bond between these described atoms basing on the catalytic mechanism. In docking, it is admitted that the affinity of the inhibitor towards lipase is related reciprocally with the ID value (when the ID value decreases the affinity increases). For CRL, we have recorded an ID of 2.7 Å (Figure 3A) which is 5 times higher than the ID recorded for HPL, with a value of 0.5 Å (Figure 3B). From these results, it is attempted that orlistat presents a strong affinity to HPL than to CRL. The ID of experimental complexes of enzyme-inhibitor for both lipases has been calculated to compare the ID and the affinities of inhibitors to each lipase. An ID of 1.6 Å has been found between the phosphorus atom of (1S)-menthyl-hexyl-phosphonate (MHP) and the oxygen atom of Ser209 for CRL [21], this value is lower than the one found for orlistat which mean that this inhibitor presented a higher affinity (Table 1, see supplementary material). For HPL, an ID of 1.7 Å has been saved between the phosphorus atom of Methoxy-Undecyl-Phosphonic acid (MUP) and the oxygen atom of Ser152 [22], when it is compared with this ID, it has been confirmed that orlistat is three times potent inhibitor than MUP (Table 1, see Supplementary material).

Conclusion:

Docking studies of the natural competitive inhibitor (orlistat) in an attempt to discover a new candidiasis treatment is of interest. Orlistat has presented a higher affinity towards HPL than to CRL, and is also a more potent inhibitor than MUP for HPL, based on ID values of both lipases. These results have shown the potential of orlistat as a new candidate for candidiasis treatment with fewer side effects in comparison to nystatin. Nonetheless, experimental *in vitro* and *in vivo* studies are required to demonstrate the effectiveness of the inhibitor. Therefore, this study illustrates affinities of inhibitors to lipases which are considerably easier to implement, cheaper and faster compared to experimental methods.

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Supplementary material:

Table 1: The inter-atomic distance values of the complexes inhibitors - lipases

Lipase Inhibitor	Orlistat	Methoxy-undecyl-phosphinic acid	(1S)-menthyl-hexyl-phosphonate
CRL	2.7	/	1.6
HPL	0.5	1.7	/

All values are in Å.