Published in final edited form as:
Microbes Infect. 2009 December ; 11(14-15): 1131–1139. doi:10.1016/j.micinf.2009.08.007.

Acetylsalicylic acid (aspirin) reduces damage to reconstituted human tissues infected with *Candida* species by inhibiting extracellular fungal lipases

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Abstract

A reconstituted human tissue model was used to mimic *Candida albicans* and *Candida parapsilosis* infection in order to investigate the protective effects of acetylsalicylic acid (aspirin, ASA). We found that therapeutic concentrations of ASA reduced tissue damage in the *in vitro* infection model. We further evaluated the lipase inhibitory effects of ASA by investigating the growth of *C. albicans*, *C. parapsilosis* and *C. parapsilosis* lipase negative (*Δcplip1-2/Δcplip1*-2) mutants in a lipid rich minimal medium supplemented with olive oil and found that a therapeutic concentration of ASA inhibited the growth of wild type fungi. The lipase inhibitors quinine and ebelactone B were also shown to reduce growth and protect against tissue damage from *Candida* species, respectively. A lipolytic activity assay also showed that therapeutic concentrations of ASA inhibited *C. antarctica* and *C. cylindracea* purified lipases obtained through a commercial kit. The relationship between ASA and lipase was characterized through a computed structural model of the Lipase-2 protein from *C. parapsilosis* in complex with ASA. The results suggest that development of inhibitors of fungal lipases could result in broad-spectrum therapeutics, especially since fungal lipases are not homologous to their human analogues.

Keywords

Candida; Acetylsalicylic Acid; Lipases; Human Fungal Pathogen; Yeast; Inhibition; Pathogenesis

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1. Introduction

Infections caused by *Candida* species manifest in a number of diseases, including candidemia, vulvovaginal candidiasis, endocarditis, and peritonitis. *Candida albicans* and *Candida parapsilosis* are two of the leading *Candida* species causing infections worldwide [1]. Risk factors for candidal infection include indwelling catheters, prosthetics, total parenteral nutrition and immuno-suppression, as in the case of individuals with HIV-AIDS, neonates, and patients receiving chemotherapy. Candidal disease is facilitated through a number of fungal virulence factors including adherence to host cells, biofilm formation, and secretion of hydrolytic enzymes, such as proteases, phospholipases, and lipases. An effective method to examine fungal virulence determinants and model initial host-pathogen interactions involves *in vitro* infections of reconstituted human tissues (RHT) [2-5]. RHT systems have been used to analyze *C. albicans* cutaneous [4] and oral [5] infections, and it has been documented that *C. parapsilosis* causes morphological damage in RHTs that mirrors those observed in human disease [3].

Lipases catalyze a number of acylation and deacylation reactions, but are best known for their hydrolysis and synthesis of triacylglycerols. The activity of these α/β-hydrolase fold family enzymes occurs via a catalytic triad composed of serine, histidine, and aspartate or glutamate residues [6]. Enzymatic activity is regulated by a “lid” or “flap” composed of an amphiphilic α-helical peptide loop that prevents substrates from reaching the catalytic triad until the enzyme encounters a lipid-water interface [6]. Such an interface induces a conformational change in the enzyme that opens the lid and allows substrate to covalently bind the active site [7]. This activation mechanism, as well as the structures of lipases isolated from many microorganisms, are highly conserved [6]. Such homology is significant as it allows lipases from diverse pathogenic organisms to break down the same energy sources used by their hosts.

An increasing amount of evidence associates lipases with microbial virulence [2,3,8-10]. Putative roles of microbial extracellular 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 [10,11]. The importance of extracellular secreted lipases has been demonstrated in *C. albicans* and *C. parapsilosis* [9,10]. Additionally, our recent work has shown that disruption of lipases attenuates damage associated with *C. albicans* [9] and *C. parapsilosis* [2] murine infections. Hence, lipase has been identified as a possible target for the development of novel anti-fungal therapeutic compounds.

Acetylsalicylic acid (ASA, aspirin) is a well-known nonsteroidal anti-inflammatory drug (NSAID) with analgesic, antipyretic and anti-inflammatory properties. The pharmacological mechanism of NSAIDs is mediated through the inhibition of cyclooxygenase (*COX*), an enzyme necessary for the biosynthesis of proinflammatory prostaglandin E_2 [12]. ASA is a nonselective *COX* inhibitor [13]. It inhibits the enzyme cyclooxygenase-1 (*COX-1*) by hindering active site accessibility by acetylating the serine-530 residue [14,15]. ASA is the only NSAID that covalently binds to a serine residue and it inhibits *COX-1* to a greater extent than cyclooxygenase-2 (*COX-2*). Although ASA's inhibition of *COX-2* proceeds through a similar mechanism, it is less effective because of the enzyme's larger and more flexible substrate channel. Aspirin has a short half life of 20 min. in circulating blood and is rapidly deacetylated and converted to salicylate, a compound that has no affect on *COX-1* or *COX-2* activity, *in vivo*.

Antifungal effects have been attributed to ASA. Aspirin suppresses morphogenesis of *C. albicans* hyphae and filamentous structures by inhibiting the formation of 3(*R*)-

hydroxyoxylipins, an oxygenated fatty acid metabolite [16]. ASA significantly decreases *C. albicans* biofilm formation and reduces viability of biofilm cells at concentrations that could be achieved in humans with therapeutic doses [17]. Additional studies found that ASA also suppresses biofilm formation of *C. guilliermondii*, *C. kefyr*, *C. glabrata*, and *C. parapsilosis* [18].

Given the previous success of RHT infection models to evaluate fungal virulence traits, we investigated the effects of therapeutic concentrations of ASA on *C. albicans* and *C. parapsilosis* pathogenicity during reconstituted human oral epithelium tissue infections. We hypothesized that ASA's protective effects were, in part, due to its ability to inhibit extracellular secreted lipase. To assess this possibility, a comparative protein model of the *C. parapsilosis* Lipase-2 in complex with ASA was constructed to examine the feasibility of interaction between the two molecules. We also investigated the effects of ASA on *C. albicans* and *C. parapsilosis* growth in lipid rich media as well as on purified lipases from *C. antarctica* and *C. cylindracea*. Salicylic acid and the lipase inhibitors quinine and ebelactone B were used in several experiments to further validate the results obtained with ASA. Quinine is an alkaloid and potassium channel blocker that is primarily used as an anti-malarial drug, but quinine also inhibits lipase [19]. Ebelactone B is a serine esterase inhibitor known to inhibit fungal lipases [3].

2. Materials and Methods

2.1 Culture media and growth conditions

The *C. albicans* and *C. parapsilosis* isolates examined are listed in Table 1. The cells were grown in YPD medium containing 1% (w/v) yeast extract, 1% (w/v) bacto peptone, and 2% (w/v) glucose. Cultures were grown in flasks for 48 h at 30°C in an orbital shaker at 180 rpm, collected by centrifugation at 2000*g*, and washed three times in PBS immediately prior to use.

2.2 Inhibition effect of ASA in a reconstituted human tissue model (RHT)

RHTs were obtained from SkinEthic Laboratory (Nice, France) and reconstituted by incubating in serum-free, MCDB 153 defined medium (Colonetics, San Diego, CA, USA), containing 5 μg/ml insulin, 1.5 mM $CaCl_2$, and 0.4 μg/ml hydrocortisone without antibiotics in 6-well tissue culture plates (Corning Inc., Corning, NY, USA) according to the manufacturer's instructions. Reconstituted human oral epithelium (RHO) was generated from human keratinocytes derived from the cutaneous carcinoma cell line TR146. Cultures were fully differentiated three-dimensional tissues, grown on the air-liquid interface for 14 days prior to delivery.

RHOs were inoculated with 2×10^6 *C. albicans or C. parapsilosis* cells in 100 μl PBS by adding the cell suspension to the 1 ml cell culture medium [2]. Control cultures were grown in medium inoculated with 100 μl PBS. The infected and control cultures were incubated at 37°C with 5% CO_2 at 100% humidity for 48 h. ASA dissolved in 0.1 M acetic acid-acetate buffer, pH 6.5, was added to inoculated and control wells at 1 mM concentration. Additionally, ebelactone B dissolved in 100% ethanol was added to wells at 150 μM. After 48 h of inoculation, tissue inserts were removed, fixed in 10% formalin for 24 h, and embedded in paraffin. Four micron vertical sections were fixed to glass slides, stained with either hematoxylin and eosin or periodic acid shiff (PAS), and examined by light microscopy.

Lactate dehydrogenase (LDH) in medium from control and experimental RHOs was measured at 48 h by CytoTox-ONE™ kit (Promega Madison, WI) according to the manufacturer's instructions. *C. parapsilosis* and *C. albicans* cells incubated in the absence of RHT were included as negative controls. The LDH released in the presence of 1 mM ASA was expressed

relative to the untreated control tissue with the LDH activity of fungal cells alone subtracted from the LDH of the infected tissue.

2.3 Effect of ASA, salicylic acid and quinine on *C. albicans, C. parapsilosis, C. parapsilosis* lipase deficient mutant growth determined by CFU

ASA at concentrations of 0.1, 0.5, 1, 2, 5 and 10 mM was dissolved in minimal medium (29.4 mM KH_2PO_4, 10 mM $MgSO_47H_2O$, 13 mM glycine, and 3 μM thiamine, sterile filtered) with 2.0% olive oil buffered by a 0.2 M citric acid − 0.1 M disodium hydrogen phosphate buffer (pH 6.5). The media was inoculated with 10^6 cells/ml of *C. albicans* or *C. parapsilosis*. Culture dilutions were made after 72 h of growth and plated on YPD agar. CFUs were taken 48 h after plating.

1 mM of ASA and salicylic acid were dissolved in minimal media with 2% olive oil buffered by a 0.2 M citric acid − 0.1 M disodium hydrogen phosphate buffer (pH 6.5). Quinine (Sigma, St. Louis, MO) was dissolved in 100% ethanol. Quinine was added to minimal media-2% olive oil to obtain final concentrations of 1 mM. Minimal media containing the drugs were inoculated with 10^6 cells/ml of *C. albicans*, *C. parapsilosis* or *C. parapsilosis* lipase knockout mutants. Control cultures included minimal media without drugs and minimal media supplemented with ethanol. The tubes were incubated at 30°C with rotary shaking at 180 rpm. Dilutions were plated on YPD agar after 24, 48, and 72 of growth. Colony forming units (CFU) were determined after 48 h of growth on YPD agar.

2.4 Lipolytic activity of commercially obtain purified *C. antartcita* and *C. cylindracea* lipase

The influence of ASA on the lipolytic activities of commercially obtained purified lipases [Fluka, St. Louise, MO] was measured via a lipase assay using α-naphthyl-palmitate as substrate with the modifications. *Candida antarctica* and *Candida cylindracea* purified lipases (0.01 units/ml) were dissolved in H_2O. 14 mM Citric Acid-74 mM Tris Buffer, pH 5.5, was added to the samples followed by 1 mM and 2 mM ASA dissolved in ethanol. 1.2 mM α-naphthyl-palmitate substrate solution dissolved in DMF was then added. The samples were agitated for 3 h at 37°C. Aliquots were collected every 30 min and the reaction was terminated by adding 0.5 M NaOH, followed by the addition of 1% Fast Violet B. Samples were gently agitated for 5 min at 37°C, then 20% Triton X100 was added and the samples were agitated for another 5 min. The samples were centrifuged at 13,000*g* for 5 min, and the lipolytic activity of the supernatants was determined photometrically by the measurement of liberated α-naphtol at 520 nm (μQuant ELISA reader). All lipolytic assays were carried out in quadruple and performed three times. The pH remained constant at 5.5 for the control and experimental tests, and pure ethanol was included as a control.

2.5 Comparative protein structure model of Candida parapsilosis Lipase-2 in complex with ASA

Comparative protein structure models were built for the only active lipase of *C. parapsilosis*, *CpLIP2*, using MODELLER. Suitable template structures were identified by running FFAS03 and PSI-BLAST programs. The resulting alignments between the templates and target as obtained from FFAAS03 and PSI-BLAST were directly used in the input for modeling. The models based on various templates and alternative alignments were energetically evaluated using the statistical scoring function implemented in the PROSA2003 program. The model based on PSI-BLAST alignment and 1ZOI template was chosen because of its overall superior energy profile and since it had the least conflicting energy scores around the location of the active site residues.

2.5 Statistics

All experiments were performed in duplicate or triplicate, and each value is shown as the mean ± SD. The significance of differences between sets of data was determined by 2-way ANOVA according to the data type.

3. Results

3.1 Inhibition effect of ASA in a reconstituted human tissue model (RHT)

Significant histological damage to the RHO inoculated with 2×10^6 cells/ml of *C. albicans* or *C. parapsilosis* occurred by 48 h after infection (Fig. 1A, B). Tissues infected with *C. albicans and C. parapsilosis* had marked attenuation of the epithelium (atrophy), epithelial whorls and a significant increase in apoptotic cells. Irregular cleft formation between cells in the upper layers with moderate disruption occurring around the surface was also observed. The basal cells were smaller and more cuboidal in shape and clefting between these cells and the spinous layers was apparent. Other observations included a decrease in intracellular bridges between spinous cells, a reduction in the size of epithelial whorls, and an absence of keratohyaline granules and mild intracellular edema within epithelial cells. Yeast cells were also adherent to the epithelial surface. Consistent with our prior work [3], tissues infected with *C. albicans* (Fig. 1A) were more severely damaged than tissues exposed to *C. parapsilosis* (Fig. 1B). More *C. albicans* cells were attached to the tissue surface, and the attached cells were characterized by a mixture of yeast, pseudohyphal, and hyphal forms.

ASA successfully attenuated the damage caused to RHO by *C. albicans and C. parapsilosis* (Fig. 1C, D). The protective effect occurred with 1 mM ASA. The lipase and kinase inhibitor ebelactone B reduced damage induced by *C. parapsilosis* at a concentration of 150 μM (Fig. 1F). Ebelactone B showed less protection against *C. albicans* infection (Fig. 1E). RHOs treated with 1 mM ASA showed no significant alterations and the concentration of LDH in supernatant was similar to that in supernatant from control tissues. LDH readings correlated with our light microscopy findings in infected tissues (Fig. 2). For *C. albicans*, 1 mM of ASA reduced LDH release by 56% in comparison to the untreated tissue infected with *C. albicans*. This concentration of ASA was even more effective than 150 μM ebelactone B, which reduced LDH activity by only 31%. Similar findings were observed for *C. parapsilosis*, where LDH activity was reduced by 79% and 39% in the case of ASA and ebelactone B, respectively.

3.2 Effect of ASA, salicylic acid and quinine on *C. albicans, C. parapsilosis, C. parapsilosis* lipase deficient mutant growth determined by colony forming units

Concentrations of 1, 2, 5 and 10 mM ASA consistently inhibited the growth of *Candida* in a concentration dependent manner (data not shown). ASA at concentrations of 0.1 and 0.5 mM inhibition of growth was not consistently significant.

1 mM ASA and quinine inhibited the growth of *C. albicans* and *C. parapsilosis* grown in buffered minimal media supplemented with 2% olive oil, although the effect of quinine was more pronounced (Fig. 3). Significant decreases in growth ($p < 0.05$) occurred at 48 and 72 h for both organisms and both drugs. Fungal cultures supplemented with ethanol or salicylic acid did not significantly reduce the growth of *C. parapsilosis* or *C. albicans* at any time point (data not shown). For statistical analyses, fungal growth in ASA was compared to growth in the media alone. The reduction in CFU due to quinine was compared to growth in media with ethanol. At no time points did 1 mM ASA, quinine or salicylic acid reduce the CFU of *C. parapsilosis* lipase disruptants (data not shown).

3.3 Lipolytic activity of commercially obtain purified *C. antarctica* and *C. cylindracea* lipase

Concentrations of 1 mM and 2 mM ASA were able to consistently reduce the lipolytic activities of the purified lipases obtained from *C. antarctica* and *C. cylindracea* in a concentration dependent manner (Fig 4A, B). After 3 hrs of incubation, 1 mM ASA was able to reduce the activity of *C. antarctica* and *C. cylindracea* by 41% and 13%, respectively, while 2 mM reduced lipolytic activity by 50% and 40%, respectively. Ethanol alone did not affect the activity of the lipases (data not shown).

3.4 Comparative Modeling of *CpLIP2* with ASA

Lipase-2 from *C. parapsilosis* (*CpLIP2*) belongs to the LIP secretory lipase family (PF03583) according to the Sanger Institute's PFAM database [20]. The LIP secretory lipase family is a member of the α/β-hydrolase fold clan. Sequence similarity search with *CpLIP2* sequence from *C. parapsilosis* using PSI-BLAST did not detect any similar structure in the Protein Data Bank [21]. In addition, no significant homology was found between *CpLIP*2 and any human lipases (665 human lipases sequence variations were selected and compared from the NCBI NR database). However, when searching the Non-Redundant sequence database [22] in general, several α/β-hydrolases were identified with known structures. Profile-profile sequence search with FFAS03 in PDB also resulted in several α/β-hydrolases (Fig. 5A). *CpLIP2* and the related known structures shared sequence identity in the range of 12-15%. Despite this low sequence relationship the active site residues of related protein structures to *CpLIP2* are fully conserved, suggesting a universal importance of the active site residues S196, D348 and, H381. Based upon the known structures obtained with FFAS03 and PSI-BLAST, a structural model of *CpLIP2* was created with ASA in the active site (Fig. 5B). Hence, ASA appears to have the capacity to intimately bind the catalytic site of *CpLIP2*.

4. Discussion

Pathogenicity arises through complex interactions between an organism's microbial virulence characteristics and the host's response. *Candida* species virulence factors include the adherence of fungal organisms to host cells, ability to form biofilms, and the secretion of hydrolytic enzymes like proteases, phospholipases and lipases [11,23]. Whereas the association of lipases to bacterial pathogenesis has been well documented [24], the significance of lipases in regards to fungal virulence remains an understudied topic that has recently gained attention. Our recent studies have underscored the importance of extracellular lipolytic activity in the virulence of *C. parapsilosis* [2] and *C. albicans* [9]. Studies of lipase inhibiting compounds, such as ebelactone B, in RHT infection models have also supported our hypothesis that extracellular lipase plays a significant role in fungal virulence [3].

We sought to further strengthen these findings by examining the capacity of ASA to attenuate damage normally associated with *C. albicans* and *C. parapsilosis* RHT infection and inhibit extracellular fungal lipase. Demonstrating that ASA can reduce fungal pathogenesis by inhibiting lipolytic activities holds significant clinical applications, particularly because lipid rich total parenteral nutrition is commonly administered to low-birth-weight and critically ill neonates, the main group at risk for infection with *C. parapsilosis* [1]. Candidal biofilms occur frequently in catheters utilized in neonates and other patients with candidemia. ASA has demonstrated potent anti-biofilm activity *in vitro* at concentrations that can be therapeutically achieved in humans and has been suggested for use in combination therapy with conventional antifungal agents in the management of biofilm-associated *Candida* infections [17]. Our data further suggest that lipase inhibitors may be used prophylactically for preventing fungal infections originating during the administration of lipid rich solutions.

ASA significantly protected RHTs during *C. albicans* and *C. parapsilosis* infections. RHO infected with *C. albicans* or *C. parapsilosis* experienced severe alterations of keratinocytes with disruption of the superficial epithelium and cleft formation between cells associated with increased intracellular edema, atrophy, and apoptosis. However, significant protection was observed at 1 mM ASA. Interestingly, the number of infecting fungal cells used in our *in vitro* infection model, 2×10^6, was much higher than the number of cells that normally initiate infection, meaning *in vivo* prophylactive applications could potentially prevent disease. LDH measurements similarly demonstrated that 1 mM ASA reduced RHO cell injury after challenge with *Candida*. Although antifungal properties have been attributed to ASA in other studies [16-18,25], its protective capabilities in an infection model have not been previously documented.

We hypothesized that ASA's protective effects were in part due to the inhibition of extracellular secreted lipase. ASA's lipase inhibition was previously demonstrated by the finding that oral administration of 3 g of ASA for 2 days to healthy subjects significantly decreases plasma free fatty acids and plasma post-heparin lipoprotein lipase activity [26]. Aspirin also inhibited the heightened lipase activity of lipoprotein lipase and hepatic lipase observed in smoking patients as measured by plasma free fatty acids and free glycerol levels [27]. We found that a 1 mM concentration of ASA consistently inhibited the growth of *C. albicans* and *C. parapsilosis* in a specific minimal medium supplemented with olive oil. Inhibition was similar to that of the lipase inhibitor quinine. In the examined lipid rich environment, organisms must rely upon secreted extracellular lipases to digest the sole energy source available. Blocking this process with lipase inhibition stalled fungal growth.

Given the wide range of effects attributed to ASA, a major concern with the data involved the possibility of our results being caused by uncharacterized or general effects of ASA. To solidify a link between ASA and lipolytic activity we used previously generated *C. parapsilosis* lipase disruptants to determine if the decrease in CFU observed in wild type candidal cells could also be documented when the two known *C. parapsilosis* lipase genes were deleted [2]. Because the growth of lipase mutants was not reduced by the addition of 1 mM ASA or quinine at any time interval tested, we conclude that ASA specifically targets wild type *C. parapsilosis* and *C. albicans* extracellular secreted lipases to impact fungal lipid acquisition. Additionally, we extended our investigation and studied the ability of ASA to inhibit purified commercial fungal lipases. Concentrations of 1 mM and 2 mM of ASA consistently reduced the activity of both *C. antarctica* and *C. cylindracea* lipase providing additional evidence that ASA has the means to inhibit fungal lipases from a number of species.

To elucidate the link between ASA and lipase we generated a computed structure model of ASA in complex with the protein *CpLIP2* and determined that the mechanism of *CpLIP2* inhibition by ASA could follow a similar mechanism to its inhibition of *COX-1* involving the acetylation of an active serine residue [12,14,15]. The catalytic triad of the lipase active site also has a serine residue which, if acetylated by aspirin, would prevent the nucleophilic residue from properly interacting with substrate. The efficacy of tetrahydrolipstatin (THL, orlistat), a potent inhibitor of human pancreatic and gastric lipases currently marketed as the over the counter drug for the treatment of obesity, alli™ (GlaxoSmithKline), supports this theory. This drug irreversibly binds the essential serine residue of the lipase catalytic triad to form an inactive acyl-enzyme in a similar mechanism to the way we believe ASA may acetylate the active serine of candidal lipases [6,28].

Salicylic acid was also used as a control to test our hypothesis for the mechanism of aspirin's lipase inhibition involving the acetylation of the active lipase serine residue. Although chemically similar as derivatives of benzoic acid, salicylic acid and ASA differ by a single functional group, an alcohol group versus an ester group, respectively. The ester group accounts

for ASAs' many documented effects, particularly its acetylation of *COX-1* serine residues. Salicylic acid is incapable of similar reactions. Hence, salicylic acid's inability to impede the growth of all three candidal strains examined supports our hypothesis and the data obtained from our computer generated structural model that ASA may acetylate the active serine residue of extracellular secreted lipase.

In summary, this work documents the protective effects of ASA in an established reconstituted oral epithelium infection model and provides a possible mechanism for the observed protection. While we do not believe aspirin should be considered a cure for candidal disease, its contribution to decreasing pathogenicity through the inhibition of lipase has serious clinical implications. Specifically, this work leaves the door open for the use of ASA, or lipase inhibitors developed in the future based upon the model of aspirin's inhibition of lipase, as prophylactic additives of lipid containing solutions, such as total parenteral nutrition, to help prevent devastating fungal infections.

Acknowledgments

JDN is supported in part by NIH AI52733, AI056070-01A2 and the Center for AIDS Research at the Albert Einstein College of Medicine and Montefiore Medical Center (NIH AI-51519). AG is supported by OTKA PD73250 and by EMBO Installation Grant 1813. AG and ZSH are supported by the János Bólyai Research Scholarship of the Hungarian Academy of Sciences.

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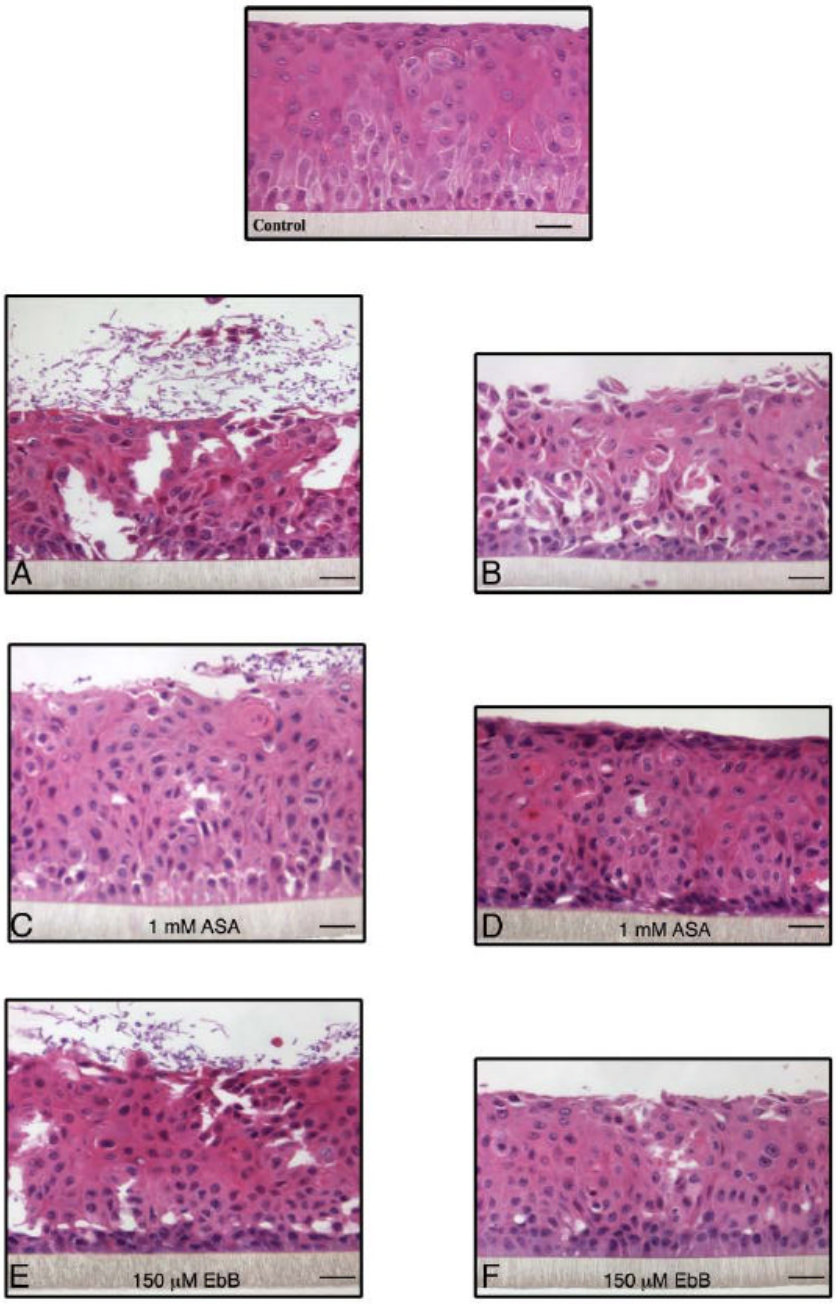

Fig. 1.

Reconstituted human oral epithelium (RHO) 48 hours after infection with *C. albicans* (A) and *C. parapsilosis* (B). Uninfected control tissue (control). RHO with *C. albicans* (C) and *C. parapsilosis* (D) in presence of 1 mM ASA. RHO with *C. albicans* (E) and *C. parapsilosis* (F) in presence of 150 μM ebelectone B.

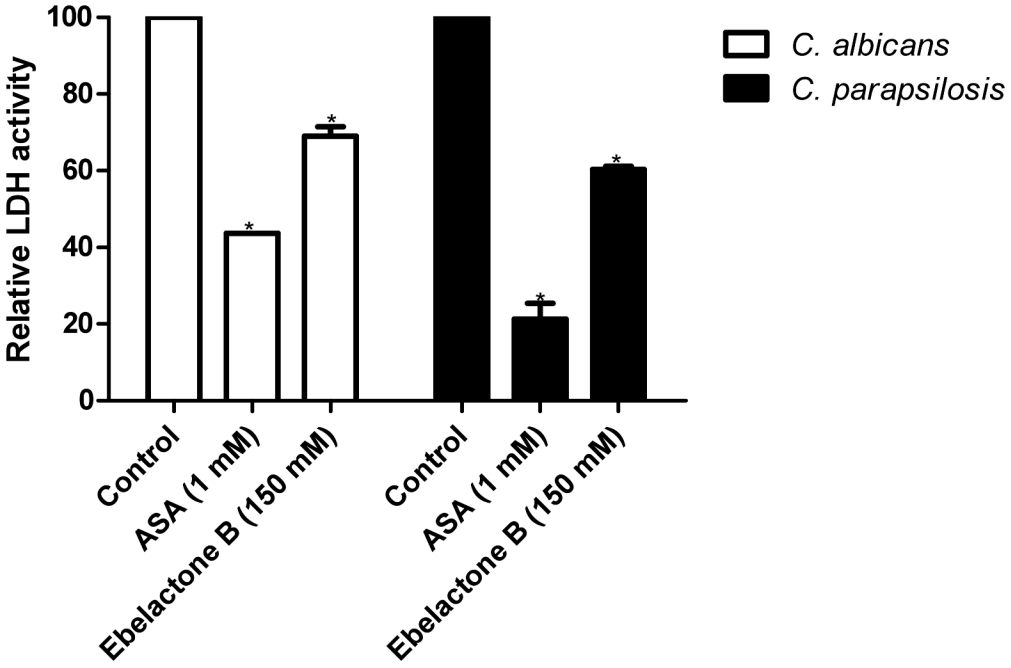

Fig. 2.
Relative LDH activity measured in tissue culture supernatant of RHO after 48 h co-culture with *C. albicans* or *C. parapsilosis*. *p<0.05.

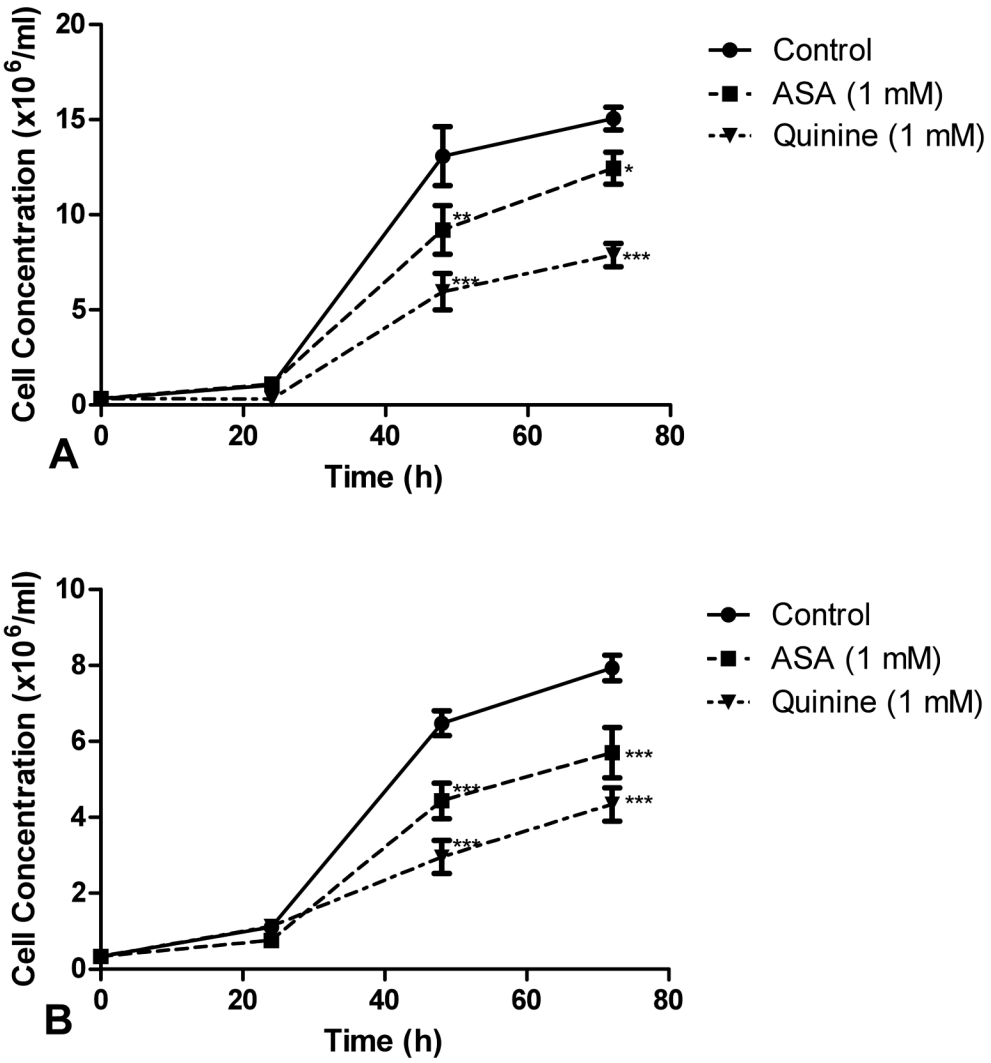

Fig. 3.

Growth curve of *C. parapsilosis* (A) and *C. albicans* (B) in buffered minimal media - 2% olive oil supplemented with ASA and quinine as determined by CFU. The data are means +/- standard deviations. *p<0.05, **p<0.01, ***p<0.001.

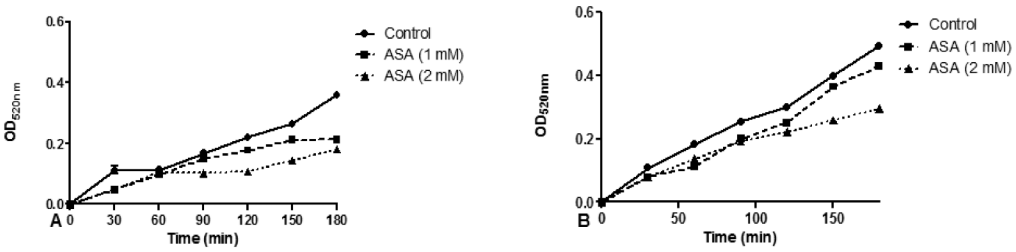

Fig. 4.

Time curve of *C. antarctica* (A) and *C. cylindracea* (B) lipase activity over 3 hours showing the effects of 1 mM and 2 mM ASA. The data are means +/- standard deviations. All values are significant ($p<0.001$).

```
_aln.pos    210       220       230       240       250       260       270
lipase2   GNLTGVSSDAETLLWGYSGGSLASGWAAAIQKEYAPELSKNLLGAALGGFVTNITATAEAVDSGPFAG
1ods      ISSFDEVDETRIGVTGGSQGGGLTIAAAALSDIP-------KAAVADYPYLSNFERAIDVALEEP---
1l7a      ISSFDEVDETRIGVTGGSQGGGLTIAAAALSDIP-------KAAVADYPYLSNFERAIDVALEQPYLE
1q0r      LDGWGV---DRAHVVGLSMGATITQVIALDHHD-------RLSSLTMLLGGGLDIDFDANIERVMRGE
1zoi      VAHLGI---QGAVHVGHSTGGGEVVRYMARHPEDKVAK------AVLIAAVPPLMVQTPGNPGGLPKS
1qtr      REMAGV---EQWLVFGGSWGSTLALAYAQTHPE-------RVSEMVLRGIFTLRKQRLHWYYQDGASR
1tht      YHWLQTKGTQNIGLIAASLSARVAYEVISDLE---------LSFLITAVGVVNLRDTLEKA-------

_aln.pos      280       290       300       310       320       330       340
lipase2   IISNALAGIGNEYPD------FKNYLLKKVSPLLSITYRLGNTHCLLDGGIAYFGKSFFSRIIRYFPD
1ods      ------------------------------------------------------------YLEINSFFRR
1l7a      INSFFRRNGSPETEV------QAMKTLSYFDIMNLA--------------------------------
1q0r      PTLDGLPGPQQPFLD------ALALMNQPAEGRAAEVAKRVSKWRILSGTGVPFDDAEYARWEERAID
1zoi      VFDGFQAQVASNRAQ------FYRDVPAGPFYGYNRPGVEASEGIIGNWWRQGMIGSAKAH-------
1qtr      FFPEKWERVLSILSDDERKDVIAAYRQRLTSADPQVQLEAAKLWSVWEGETVTLLPSRESASFGEDDF
1tht      ---------------------LGFDYLSLPIDELPNDLDFEGHKLGSEVFVRDCFEHHWDTLDSTLDK

_aln.pos        350       360       370       380       390       400
lipase2   GWDLVNQE----PIKTILQDNGL-VYQPKDLTPQIPLFIYHGTLDAIVPIVNSRKTFQQWCDWGLKSG
1ods      NGSPETEV----QAMKTLSYFDI-MNL--AYRVKVPVLMSIGLIDKVTPPSTVFAAYNHLET----KK
1l7a      ------------------------------DRVKVPVLMSIGLIDKVTPPSTVFAAYNHLET----KK
1q0r      HAGGVLAE----PYAHYSLTLPPPSRAAELREVTVPTLVIQAEHDPIAPAPHGKHLAGLI-----PTA
1zoi      -------------YDGIVAFSQT-DFTEDLKGIQQPVLVMHGDDDQIVPYENSGVLSAKL----LPNG
1qtr      ALAFARIENHYFTHLGFLESDDQ-LLRNVPLIRHIPAVIVHGRYDMACQVQNAWDLAKAW-----PEA
1tht      ----------------------------VANTSVPLIAFTANNDDWVKQEEVYDMLAHIRT---GHC

_aln.p  410       420       430       440       450       460       470
lipase2   EYNEDLTNGHITESIVG---APAALTWIINRFNGQPPVDGCQHNVRASNLEYPGTPQSIKNYFEAALH
1ods      ELKVYRYFGHEYI-PAF---QTEKLAFFKQHLK----------------------------------
1l7a      ELKVYRYFGHEYI-PAF---QTEKLAFFKQILKG---------------------------------
1q0r      RLAEIPGMGHALPSSVHGPLAEVILAHTRSAA-----------------------------------
1zoi      ALKTYKGYPHGMPTTHADVINADLLAFIRS--------------------------------------
1qtr      ELHIVEGAGHSYDEPGI---LHQLMIATDRFAG-----------------------------------
1tht      KLYSLLGSSH--DLGEN---LVVLRNFYQSVTK-------------AAIAMDGGSLEIDVDFIEPDFE

 _aln.pos  480       490       500
lipase2   AILGFDLGPDVKRDKVTLGGLLKLERFAF
1ods      -----------------------------
1l7a      -----------------------------
1q0r      -----------------------------
1zoi      -----------------------------
1qtr      -----------------------------
1tht      QLTIATVNERRLKAEIENRTPE-------
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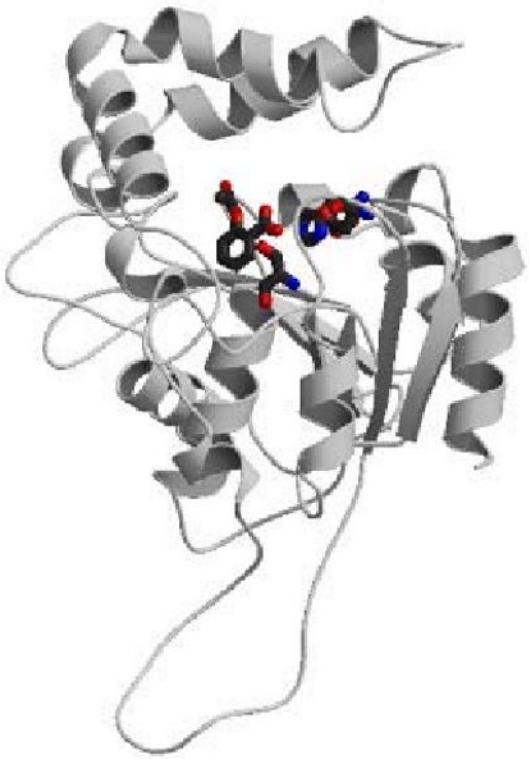

Fig. 5.

Fig. 5A. A fragment of the multiple sequence alignment obtained from fold recognition program FFAS03. All active site residues are part of the shown fragment (in bold red). The query protein Lipase-2 is in the first line, similar proteins with known 3D structures are aligned underneath. Four letter PDB codes are shown for each protein.

Fig. 5B. Structure of *CpLIP2* in association with ASA obtained with FFAS03 and PSI-BLAST.

Table 1

Clinical isolates used in this study

Organism	Reference
Candida albicans SC5314	Gillum et al. (1984) [29]
Candida parapsilosis GA1	Gacser et al., (2005)[30]
Candida parapsilosis Lipase KO, Δ*cplip1-2*/Δ*cplip1-2*	Gacser et al., (2007) [2]