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Preclinic study of Bgl2 ligand as component of mix antibiofilm toward *Candida albicans* on mucous intestinal biofilm preinduced *Rattus novergicus*

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ABSTRACT

Candida biofilms are difficult to be penetrated by drug molecules and resistant to almost all types of antifungal (multi drug resistance), included fluconazole. The successfull of *Candida* treatment is determined by eradication of extracellular matrix of *Candida* biofilms, especially which attach in the gastrointestinal tract of patients. In the previous studies it was successfully eradicated *Candida* biofilms in vitro using novel biomaterials that are in the process of patent, those are the combination of hydrolase enzyme and ligand Bgl2 used to hydrolyze the extracell matrix and to prevent the matrix regeneration respectively. The effects were proved by increasing of fluconazole activity as much as 61.14%. The purpose of this research were determining the effectiveness and ED₅₀ of ligand Bgl2 as the antibiofilm candidate toward *Candida* biofilm extracellular matrix in mice as the animal models. Research methods included the formation of *Candida albicans* biofilms on the mucous membran of gastrointestinal tract of mice, followed by healing it using mix antibiofilm: hydrolase enzymes and ligand Bgl2, to elevate the action of fluconazole. The concentration of Bgl2 ligand were varied and percent inhibition of biofilm formation was determined for each dose. The reduction of the biofilm as responds to the treatments were analyzed to determine the ED₅₀. The biofilm were analyzed by determining of *Candida* viability (CFU) in faecal and intestinal tissue samples for several weeks of biofilm induction, and SEM analysis of the biofilm sample. The elevated doses of Bgl2 ligand 0, 5, 10, 15, 17,5 and 20 mg/kg body weight gave the percent inhibition of biofilm formation 0, 31, 54, 93, 100 and 69 % respectively, from which was calculated the ED₅₀ of Bgl2 ligand for *C. albicans* eradication was 8,57 mg /kg body weight. Hydrolase enzymes and ligand Bgl2 effectively enhanced the fluconazole activity on two weeks treatment. ED₅₀ Bgl2 ligand value of 8,57 mg / kg body weight is smaller than those LD₅₀ reported 17,5 mg / kg body weight. This means that biofilm eradication using combination of antibiofilm found in this research to eradicate *C. albicans* biofilm does not cause side effects. The conclusions were: (1) ED₅₀ of Bgl2 ligand was 8,57 mg/ kg body weight on using in combination with hydrolase enzymes and fluconazole (mix antibiofilm) (2) Hydrolase enzymes and ligand Bgl2 effectively increased the fluconazole activity.

Keywords: mix antibiofilm, hydrolase enzyme, Bgl2 ligand, ED₅₀

Abbreviations

Bgl2	= Beta glucanase 2
ED ₅₀	= Effective dose fifty
LD ₅₀	= Lethal dose fifty
SEM	= Scanning electron microscopy
CFU	= Colony forming unit
Kg	= Kilogramme
mg	= Milligramme
<i>C. albicans</i>	= <i>Candida albicans</i>
SAPs	= Secreted aspartyl proteinases
Glu	= Glucose
kDa	= kilo Dalton
DNA	= Deoxyribo nucleic acid
PBS	= Phosphate buffer saline

INTRODUCTION

Overgrowth of *Candida* spp. and the biofilm formation in the human intestinal tract are a crucial but negligible chain increasing incidence of many diseases today, especially degenerative diseases. At least nine lipase isozymes can be produced by *C. albicans*, hydrolyzing ester bonds of mono-, di-, and triacylglycerols [1]. Hydrolytic enzymes named SAPs also secreted by *C. albicans*. At least ten different genes, *SAP2*–*SAP10* were reported encoded SAP isozymes [2]. These aspartyl proteinases hydrolyze host proteins including collagen, laminin, fibronectin, mucin, salivary lactoferrin, α 2-macroglobulin, almost all immunoglobulins, the proinflammatory cytokine interleukin-1 β , lactoperoxidase, cathepsin D, complement, cysteine A, and precursors of several blood coagulation factors [2, 3]. The optimal pH of SAPs activity range from 2.0 to 7.0, therefore these enzymes contribute to fungal pathogenesis and disseminate infections at different sites in the human organism [3]. *C. albicans* also produce other secretory proteases: a 60-kDa metalloproteinase and a 50-kDa serine peptidase. The serine peptidase is active in a broad range of pH (5.0–7.2) and hydrolyzes many host proteins including extracellular matrix proteins and serum proteins [4]. *Candida* spp. also produce a variety of toxic metabolites, some of which interfere with the immune system. Therefore, when the infection is not immediately cured, will lower the human immunity in the site of colonization and causes complications and degenerative diseases.

High amount of primary and secondary metabolites produced by *Candida* spp. are harmful to the body, which is currently happening in the *Candida* overgrowth and biofilm related diseases. The toxic metabolites of *Candida* spp. includes ethanol, formaldehyde, acetaldehyde, D-arabinitol, and organic acid such as tartaric acid. Some *Candida* spp. metabolites are denaturants, causing denaturation of host protein molecules. There is also a metabolite of *Candida* spp. that cause DNA mutations, resulting misfold and conformational changes in the protein molecule. Proteins are part of the molecular machinery of cells which is very important components. While protein is very sensitive to the denaturant compounds. Structural or conformational abnormalities due to the presence of protein denaturant compounds resulting protein dysfunction, named conformational diseases or degenerative diseases. Candidiasis and dysbiosis prevalently found in children with Autistic Spectrum Disorders [5]. Around ten last year was understood that a large number of diseases with very different pathology, at the cellular level can be studied in the frame of protein misfolding, which include Alzheimer's disease, Parkinson's, prion, type 2 diabetes, amyloidosis, cystic fibrosis, sickle cell anemia, cataracts, and others [6, 7].

The formation of *Candida albicans* biofilm has been demonstrated in the previous *in vitro* study [8]. We also have notified the formation of pathogenic *Candida albicans* biofilm *in vivo* on mucous membrane of *Rattus norvegicus* intestinal tract [9]. The *in silico* study found a conformational antibiotic "X" as the best ligand for Bgl2 enzyme, which was acting as inhibitor. This research obtained that "X" showed similarity structure with β -D-glucose, with the binding affinity -7.71 kcal/mol and the inhibition constant 2.23 μ M. It was identified that "X" interacted with Glu192 and Glu292 which are catalytic residues of Bgl2 enzyme. It was proved that the antifungal activity of fluconazole increased highly with existence of the hydrolytic enzyme extract and "X". Further more the biofilm formation highly decreased by 91.43% at 3 hours of incubation and by 91.35% after 6 hours. SEM analysis of the treated biofilm showed that the extracellular matrix completely run out and debris cell appeared as disunion hyphae. In the present article we report the ED₅₀ of the Bgl2 ligand.

MATERIALS AND METHODS

Materials used in this experiment were hydrolytic enzymes extract & Bgl2 ligand from the previous research [9], *Candida albicans* and *Rattus norvegicus* strain Wistar. Bgl2 ligand was an antibiotic, and fluconazole were purchased from pharmacy. *Candida albicans* strain was obtained from the culture collection of the microbiology research division, Airlangga University, Indonesia. The preparation of planctonic *Candida albicans* were described in the previous article [8]. *Rattus norvegicus* used as testing animal. Total testing animals were 30, divided into 2 groups each consist of 15 mice. They were randomly taken to be the control and the treatment groups. Adaptation period for each group was 2 weeks. After the adaptation period is completed the animals test entered the treatment period for induction of intestinal biofilm.

Preparation of *Candida albicans* inoculum

The stock culture of *Candida albicans* was grown on Sabouraud dextrose agar (SDA) at room temperature. The single colony was inoculated on YPD broth by shaking on rotary shaker 160 rpm overnight. The cell pellet was removed by centrifugation at 10,000 rpm, 4 °C, 15 minutes. The pellet was washed twice using sterile phosphate saline buffer (PBS) 0.1 M, and was suspended in PBS.

Induction of intestinal *Candida albicans* biofilm of *Rattus novergicus* strain Wistar

Intestinal biofilm formation were done according to the previous research [9]. Experimental animals were divided into 2 groups, control and treatment groups. The control group to be sacrificed in order to observe macroscopically *Candida albicans* biofilm on the mucous membrane of the small intestine and cecum after induction treatments. Treatment groups was not sacrificed after the control group showed biofilms on the intestinal mucous, but followed by treatment with mixture of enzyme extract, Bgl2 ligand and fluconazole for biofilm eradication experiment. Each animal group received the same treatment as follows. Animal were fed 2 ml of *Candida albicans* inoculum with OD 0.5, after took the antibiotics tetracycline, streptomycin, and gentamycin. All animals were took antibiotics for 4 days prior to inoculation. In addition to antibiotics, cortisone acetate was injected subcutaneously the day before.

Identification of biofilm

Identification of intestinal biofilm was done by several ways, the first were macroscopic observations of mucous membran of the small intestine and cecum after splitting it by magnifying lens. Second, biofilm identification by using SEM. Third, determination of *C. albicans* cells number in the biofilm by using Total Plate Count method, then calculated the cell free unit (CFU).

Candida albicans cell in faecal samples, small intestine and cecum were determined by the method Total Plate Count. Faecal sampling was carried out for a maximum of 10 minutes. Dilution series of samples, each subsequent suspension grown on YPD agar medium containing 50 mg ampicillin / ml and 100 mg streptomycin / ml, were grown for 24 hours at room temperature.

Determination of ED50 Bgl2 ligand for intestinal biofilm eradication

Rattus novergicus harbouring intestinal biofilm were treated with antibiofilm consist of hydrolitic enzyme extract, Bgl2 ligand (compound "X") and fluconazole. The blood serum were collected from the test animals periodically after and during treatment with antibiofilm until the biofilm vanished.

RESULTS AND DISCUSSION

After *Candida albicans* biofilm has been observed by using SEM on mucous membranes of rats GI, the animals then treated to eradicate the biofilm for 2 weeks. The evidence of losing biofilm was observed by measuring the viability of *Candida albicans* in the faecal samples and intestinal tissue in the first and second weeks. Inhibition of biofilm formation by mix biofilm with variation of Bgl2 ligand concentration in Table 1.

Table 1. Percentages of biofilm inhibition by mix antibiofilm with variation of ligand Bgl2 doses

No	Ligand Bgl2 (mg/kg body weight)	Candida viabilities (CFU / mL)		The average of Candida viabilities (CFU / mL)		% Inhibition of biofilm formation
		Before therapy	After therapy	Before therapy	After therapy	
1	0	183.05 172.00 193.10	183.05 172.00 193.10	183	183	0
2	5	183.05 172.00 193.10	126.76 123.81 129.71	183	127	31
3	10	183.05 172.00 193.10	67.74 101.61 No data	183	85	54
4	15	183.05 172.00 193.10	18.99 9.37 13.43	183	14	93
5	17,5	183.05 172.00 193.10	0 0 0	183	0	100
6	20	183.05 172.00 193.10	40.00 78.95 55.00	183	57.98	69

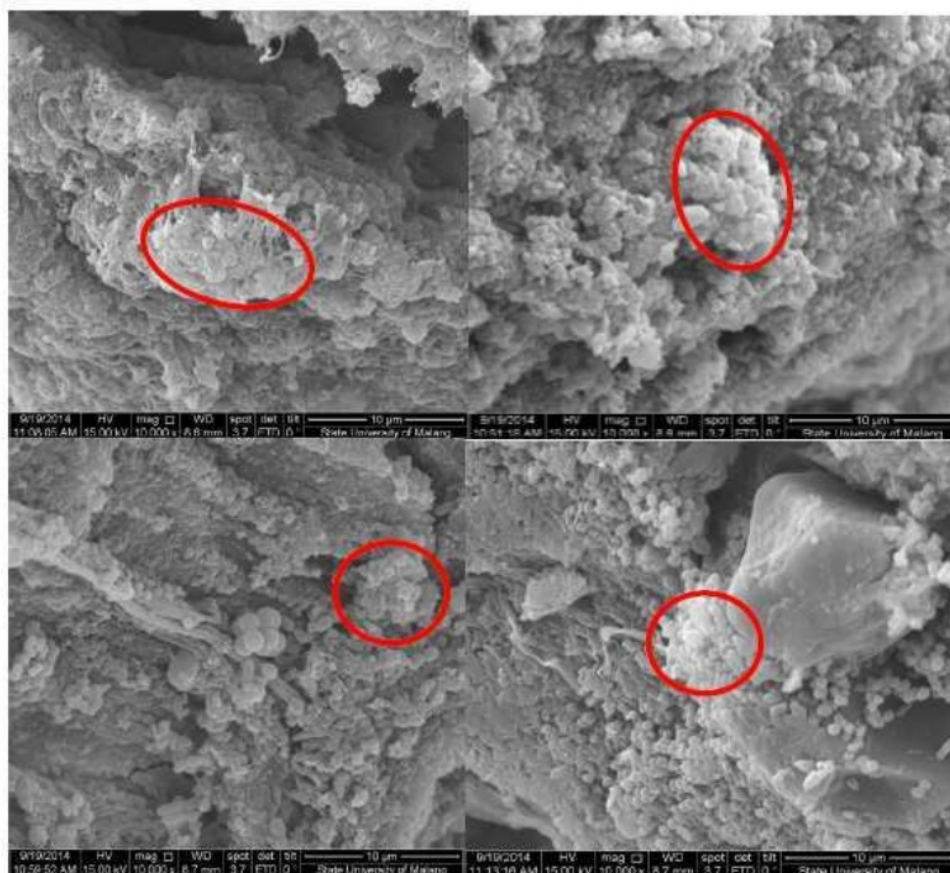


Figure 1. SEM profile of intestinal *Candida albicans* biofilm without antibiofilm therapy. The thickest matrix extracellular of *Candida albicans* biofilms were circled in red in the each field of view (magnification: 10.000 x)

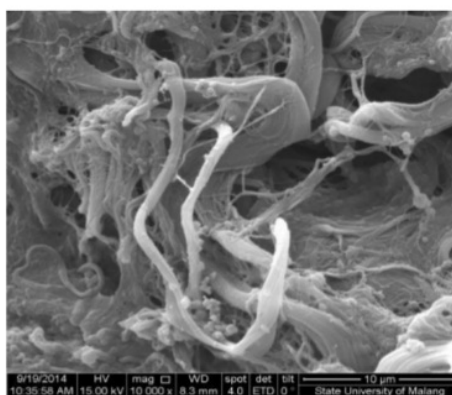


Figure 2. SEM profile of intestinal membran of *Rattus norvegicus* after antibiofilm therapy. Biofilm or planctonic *C. albicans* were eradicated (magnification: 10.000 x)

According to Table 1, the percent inhibition of biofilm increased with increasing the Bgl2 ligand doses. The total inhibition occurred at 17.5 mg / kg body weight. At 20 mg / kg body weight the percent inhibition decreased. It was likely due to the decrease in enzyme activity. Thus the data percent biofilm inhibition at 20 mg / kg body weight was not used for the calculation of ED50.

Statistical analysis of the data on Table 1 using SPSS program obtained ED50 value of 8.57 mg / kg body weight. The inhibitioncurve profile in Figure 3.

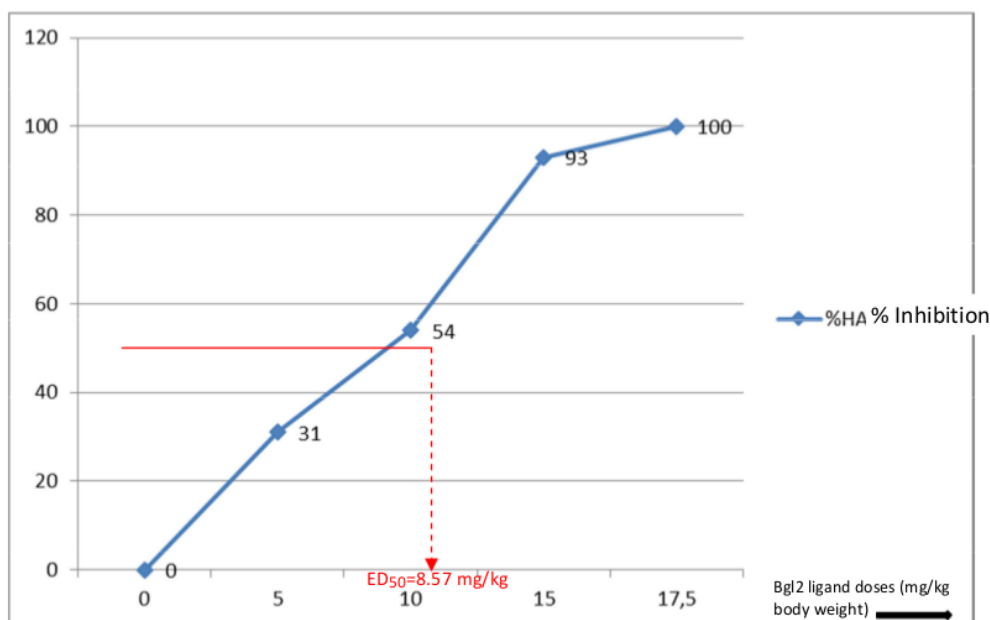


Figure 3. The inhibition profile of intestinal *Candida albicans* biofilm toward mix antibiofilm with Bgl2 ligand doses variation

Bgl2 (beta glucan2) enzyme is one of the cell wall constituent of *Candida albicans*. This is a polymerase enzyme that synthesis β -1,3 and β -1,6 glucan from its substrate glucose. The glucans are the major component of matrix biofilm that protect the pathogenic *Candida* from antifungals and body immune system. Bgl2 ligand "X" is small molecule that compete with the substrate glucose to occupy the active side of Bgl2. This leads to the inhibition of the glucans formation by Bgl2 enzyme [8]. The novel activity of "X" is our finding in the previous research. Nevertheless, compound X is conventional medicine with anti-bacterial properties. Mechanism of the antibacterial activity was through the inhibition of protein biosynthesis. On the orally use showed LD50 (mouse) 1520 mg / kg body weight.

Fluconazole alone can not eliminate hidden *Candida* cells covered by matrix extracellular in the biofilm form [8]. The use of enzymes without Bgl2 ligand also less effective for improving the performance of fluconazole, because the ability of *Candida* to regenerate the extracellular matrix. Combination of hydrolytic enzymes omitting matrix extracellular and Bgl2 ligand which suppress the extracellular matrix regeneration, have been proved effectively improving the performance of conventional antifungal like fluconazole.

CONCLUSION

The ED50 of Bgl2 ligand was 8.57 mg / kg body weight on used in combination with mix antibiofilm. This value was much smaller than LD50 previously reported (1520 mg / kg body weight). This indicates that biofilm eradication with our finding mix antibiofilm are expected to work effectively without causing serious side effects.

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