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Brief Report

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Anti-apoptotic effects of decyl gallate on the induction of apoptosis in A549 pneumocytes by *Paracoccidioides brasiliensis* gp43

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Abstract

Apoptosis is considered an escape mechanism from the host immune system for the fungus *Paracoccidioides* spp, and it serves as a vehicle for entry into macrophages without stimulating microbicidal activities. Recently, gp43 of *P. brasiliensis* was demonstrated to be involved in this process. Therefore, as a new therapeutic alternative, it is very important to study compounds that could reduce the modulation of the induction of apoptosis caused by this fungus. Decyl gallate (G14) is a known antifungal compound, and we decided to investigate its anti-apoptotic properties. Our results demonstrate that G14 was effective against apoptosis induced by gp43, as observed in epithelial cells, and led to a reduction in DNA damage, Bak down-regulation and Bcl-2 up-regulation. Together, these data show that G14 presents promising anti-apoptotic activity.

Key words: *Paracoccidioides brasiliensis*, gp43, apoptosis, decyl gallate.

Introduction

Paracoccidioidomycosis (PCM) is a systemic mycosis that is distributed from Mexico to Argentina, with the highest prevalence in Brazil.[1] The infection occurs by inhalation of conidia and/or hyphae fragments of *Paracoccidioides* spp.[2]

These fungi acts through several mechanisms, including the induction of apoptosis to evade the immune

system and proliferate[3,4] that involve members of the Bcl-2 family, which comprise pro-apoptotic (Bak, Bax, Bim) and anti-apoptotic (Bcl-2,B cl-1) proteins.[5] *Paracoccidioides* could induce apoptosis, increasing the expression of pro-apoptotic proteins,[6] and gp43 is able to trigger apoptosis in peripheral blood mononuclear cells[7] and in pneumocytes.[8]

The search for new antifungals is necessary, and De Paula e Silva et al.[9] showed that decyl gallate (G14) possesses low toxicity and great fungicide activity against several pathogenic fungi, including *Paracoccidioides* spp. In this study, we showed that in addition to fungicide activity, G14 also prevents the induction of apoptosis by *P. brasiliensis*, reinforcing the potential use of this molecule against PCM.

Methods

Fungal strain, growth conditions and gp43 preparation

P. brasiliensis (Pb18 isolate) yeast cells were cultivated in Fava-Netto[10] medium at 37°C. The gp43 protein was purified from Pb18 exoantigen using gel filtration chromatography.[11]

Cell lineage

Pneumocytes A549 (RJ Cell Bank) were seeded in HAM-F12 medium (Cultilab, Brazil) supplemented with 10% heat-inactivated fetal bovine serum and incubated at 36.5°C with 5% CO_2 for 24 h for the formation of a cell monolayer.

Antifungal substance: decyl gallate (G14)

Decyl gallate (G14) derived from gallic acid at the minimum inhibitory concentration of 0.012 μmol[9] was used in this study.

A549 monolayer formation and treatment with gp43 and G14

The A549 monolayer was prepared at 36.5°C with 10^5 cells/well and incubated for 24 and 48 h with 50 μg/ml gp43 or gp43+G14. As a negative control, A549 cells without treatment (NoTx) were employed. After incubation, the cells were washed with phosphate-buffered saline (PBS) and then used in the assays.

Comet assay

DNA damage was evaluated by comet assay for 24 h, according to Singh et al.[12] As a positive control, the cells were treated with 0.001 M hydrogen peroxide, and as a negative control, we used NoTx. The slides were stained with ethidium bromide (20 mg/ml) (Sigma Aldrich) and visualized with a fluorescence microscope (EVOS fl AMG; Westover Scientific). The images of 100 random nucleotides from each replicate were analysed using Comet Score software (TriTek).

Table 1. Primers used for the real-time PCR assays.

Gene	Primer sense (5′→3′)	GenBank
Bak R	CTGCCAGGGAACAGAGAAGG	U237651
Bak F	GTTCTGTTGGGCCAGTTGT	
Bcl-2 R	CCTTGGCATGAGATGCAGGA	NM-00657
Bcl-2 F	GATTGATGGGATCGTTGCCTTA	
GAPDH R	TGGTGAAGACGCCAGTGGA	NM-002046
GAPDH F	GCACCGTCAAGGCTGAGAAC	

Evaluation of Bak and Bcl-2 protein expression by flow cytometry

For these assays, after treatment, the cells were removed, fixed in 70% ethanol and permeabilized (1% saponin), followed by incubation with anti-Bak or anti-Bcl-2 (1:100) antibodies for 1 h. The cells were incubated with FITC-labelled anti-mouse antibody for 30 min. The cells (10,000/sample) were analyzed by BDFACSCanto flow cytometry, and the analysis was performed using FACSDiva software (Becton Dickinson, USA).

Transcriptional changes in the apoptotic/anti-apoptotic markers by real-time PCR (qPCR)

Alterations in the mRNA expression of genes involved in the induction and prevention (Bak and Bcl-2, respectively) of apoptosis were evaluated by quantitative polymerase chain reaction (qPCR) using specific primers (Table 1).

After treatment, RNA extraction was performed using the TRIzol method (Invitrogen Life Technologies, USA), followed by cDNA synthesis using RevertAidTM H Minus Reverse Transcriptase (Thermo Scientific, USA). The qPCR reactions were carried out using 1× Maxima® SYBR Green/Rox qPCR Master Mix (Thermo Scientific, USA) using the Applied Biosystems 7500 thermocycler. The results were expressed relative to the housekeeping gene GAPDH and analysed by the $2^{-\Delta\Delta CT}$ method.[13] The qPCR reactions were performed in triplicate in three independent experiments.

Statistical analysis

Statistical analysis was calculated by ANOVA, Tukey's test using GraphPad Prism 5.0 software. The differences were considered statistically significant at $P < .05$.

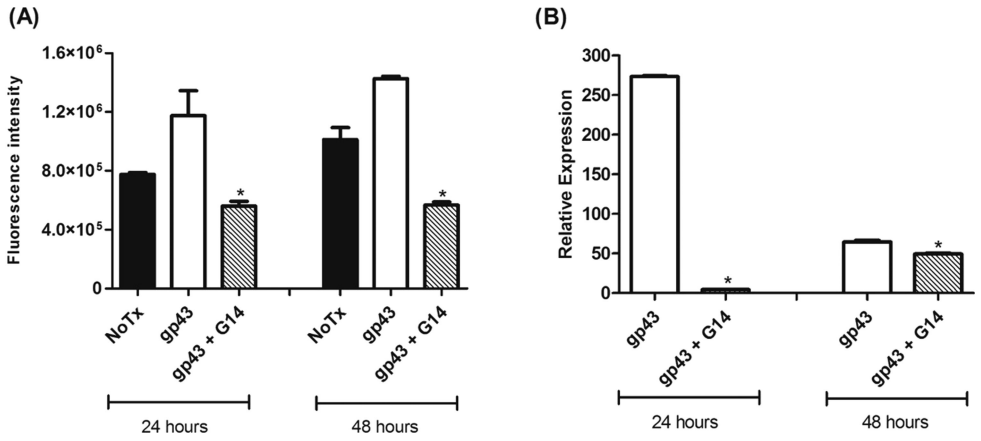

Figure 1. Expression of pro-apoptotic marker Bak in A549 pneumocytes. Evaluation by flow cytometry (A) and qPCR (B) in A549 cells without treatment (NoTx); treated only with gp43 and cells treated with gp43+G14 after 24 and 48 h of incubation. $^{*}P < .05$.

Results

The capacity of G14 to avoid DNA damage in pneumocytes caused by *P. brasiliensis* gp43

As expected, the positive control showed a high DNA damage index (40%). Gp43 led to 45% DNA fragmentation, which was a 5% increase compared to the positive control. However, the treatment with gp43+G14 led to a significant decrease, approximately 10%, compared to the value obtained for the gp43 treatment, with the DNA fragmentation index reduced to 35% (data not shown).

The influence of gp43 and G14 on the expression of the pro-apoptotic marker Bak

The cells treated with gp43 had increased Bak expression by 38.5% and 23% after 24 and 48 h, respectively, when compared to NoTx. Moreover, cells treated with gp43+G14 showed a significant reduction (50%) in the expression of Bak after 24 and 48 h compared to the cells that were only treated with gp43 (Fig. 1A). Using qPCR, the treatment of A549 cells with gp43 for 24 h promoted 271-fold higher the expression of Bak, while when treated with gp43 combined with G14 we only observed 17.7-fold. After 48 h, we observed 52.5-fold of Bak expression when treated with gp43 and 39-fold when gp43 was combined with G14. Significant influence of G14 was observed in downregulate the expression of the pro-apoptotic marker Bak (Fig. 1B).

Influence of gp43 and G14 on the expression of the anti-apoptotic marker Bcl-2

The cells treated with gp43 for 24 and 48 h showed a decrease in Bcl-2 expression, demonstrating the loss of anti-apoptotic signalling. Treating the cells with gp43+G14 did not change the expression of Bcl-2, indicating the cells were protected from the effect caused by gp43 (Fig. 2A). These results were confirmed by qPCR, from which we also observed a decrease in the expression of Bcl-2 after 24 and 48 h of treatment. When the cells were treated with gp43+G14, we observed an increase in the expression of the Bcl-2 gene by 2.73-fold after 24 h. After 48 h, we could not observe any differences between the expression levels of cells receiving different treatments (Fig. 2B).

Discussion

Apoptosis, programmed cell death of great importance in the development and homeostasis of multicellular organisms, has been described in microbial pathogenesis and involves a variety of host-pathogen interactions.[14,15] While apoptosis is essential for multicellular organisms, it is also associated with a wide range of diseases and pathological conditions.[16,17] During its host-cell interactions, *P. brasiliensis* induces apoptosis,[6] and two of its the most important adhesins, gp43 and 14-3-3, may play a role in these mechanisms because they also have the capacity to induce cellular apoptosis.[8] However, alkyl gallates have a wide range of biological activities, including anti-*Paracoccidioides* activity,[9] the inhibitory effects of collagenase and hyaluronidase[18] and the loss of mitochondrial membrane potential.[19] Thus, in this work, we assess the anti-apoptotic potential of G14.

We observed that G14 inhibited or reduced the damage to cellular DNA caused by gp43 in pneumocytes. Cells treated with G14 for 24 h showed a decrease in DNA damage, suggesting its anti-apoptotic potential, which was reinforced through the Bak and Bcl-2 expression analysis. The treatment of cells with gp43 leads to a significant increase in Bak expression, which induces the activation of apoptosis. However, when G14 was also used in the treatment, Bak

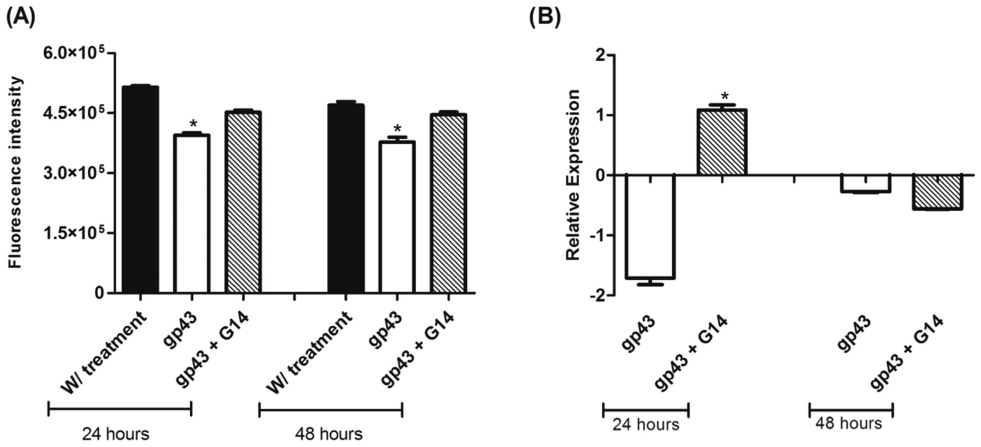

Figure 2. Expression of anti-apoptotic marker Bcl-2 in A549 pneumocytes. Evaluation by flow cytometry (A) and PCR real time (B) in A549 cells without treatment (NoTx); treated only with gp43 and cells treated gp43+G14 after 24 and 48 h of incubation. $^{*}P < .05$.

expression was significantly reduced, protecting the cells from apoptosis. In the case of Bcl-2 expression, our results showed that the treatment of cells with gp43 after 24 and 48 h led to a decrease in its expression, which implies a reduction in anti-apoptotic signalling, whereas when G14 was also used, we did not observe any difference in Bcl-2 expression compared to the NoTx control, indicating that G14 protects the cells from apoptosis induced by gp43.

The apoptosis process is a complex event, and different pathways can be activated. It can be initiated by two main signalling pathways: mitochondrial (intrinsic pathway) and death receptors (extrinsic pathways). The intrinsic pathway involves the member of Bcl-2 family that consists in apoptotic (Bak, Bax, Bim, and BclXS) and anti-apoptotic (Bcl-2, BclXL, and Bcl-1) proteins. The apoptosis initiates by the activation of apoptotic proteins, triggered by DNA damage for example, which promotes the disruption of mitochondrial outer membrane, that leads to caspase-9 cleavage resulting in the activation of effector caspases promoting cell death. It has already been shown that *P. brasiliensis* gp43 acts on the members of Bcl-2 family and caspases[6,8,20] that are important elements of the mitochondrial pathway. Regarding the gallates, it has been demonstrated that these molecules can modify mitochondrial membrane potential in *T. cruzi*.[19] In this way, since gallates decrease the mitochondrial membrane potential, that is one of the action targets of gp43 in the induction of apoptosis, we suggest that the process would be suppressed by the gallates as demonstrated in this study. However, additional studies focusing specifically on mitochondrial markers should be developed.

Therefore, the search for substances that modulate or participate in this host-pathogen interaction may be important for a better understanding of the pathogenesis of this fungus and to develop new strategies to fight fungal infections in the future. This study showed that along with the fungicide action, G14 also acts in the host cells to prevent the induction of apoptosis by *P. brasiliensis*, reinforcing its potential use in PCM therapy and studies *in vivo* are in progress with this drug and should be of great importance to better characterize the action of this drug in fighting PCM.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

References

1. Bonfim SM, Cruz AH, Jesuino RS et al. Chitinase from *Paracoccidioides brasiliensis*: molecular cloning, structural, phylogenetic, expression and activity analysis. *FEMS Immunol Med Microbiol*. 2006; 46: 269–283.
2. Takayama A, Itano EN, Sano A et al. An atypical *Paracoccidioides brasiliensis* clinical isolate based on multiple gene analysis. *Med Mycol*. 2010; 48: 64–72.
3. Mendes-Giannini MJ, Hanna SA, da Silva JL et al. Invasion of epithelial mammalian cells by Paracoccidioides brasiliensis leads to cytoskeletal rearrangement and apoptosis of the host cell. *Microbes Infect*. 2004; 6: 882–891.
4. Sridharan H, Upton JW. Programmed necrosis in microbial pathogenesis. *Trends Microbiol*. 2014; 22: 199–207.
5. Donovan M, Cotter TG. Control of mitochondrial integrity by Bcl-2 family members and caspase-independent cell death. *Biochim Biophys Acta*. 2004; 1644: 133–147.
6. Del Vecchio A, Silva JeF, Silva JL et al. Induction of apoptosis in A549 pulmonary cells by two Paracoccidioides brasiliensis samples. *Mem Inst Oswaldo Cruz*. 2009; 104: 749–754.

7. Ferreira KS, Almeida SR. Immunization of susceptible mice with gp43-pulsed dendritic cells induce an increase of pulmonary Paracoccidioidomycosis. *Immunol Lett*. 2006; 103: 121–126.
8. Silva JF, Vicentim J, Oliveira HC et al. Influence of the Paracoccidioides brasiliensis 14-3-3 and gp43 proteins on the induction of apoptosis in A549 epithelial cells. *Mem Inst Oswaldo Cruz*. 2015; 110: 476–484.
9. Alves de Paula e Silva AC, Costa-Orlandi CB, Gullo FP et al. Antifungal activity of decyl gallate against several species of pathogenic fungi. *Evid-Based Complement Alterna Med*. 2014; 2014: 506273.
10. Fava Netto C. *Contribuição para o estudo imunológico da blastomicose de Lutz (Blastomicosesulamericana)*. São Paulo: Rev Inst A Lutz; 1961; 21: 99–194 [in Spanish].
11. Mendes-Giannini MJ, Bueno JP, Shikanai-Yasuda MA et al. Detection of the 43,000-molecular-weight glycoprotein in sera of patients with paracoccidioidomycosis. *J Clin Microbiol*. 1989; 27: 2842–2845.
12. Singh NP, McCoy MT, Tice RR et al. A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp Cell Res*. 1988; 175: 184–191.
13. Wüthrich M, Deepe GS, Klein B. Adaptive immunity to fungi. *Annu Rev Immunol*. 2012; 30: 115–148.
14. Villar CC, Zhao XR. Candida albicans induces early apoptosis followed by secondary necrosis in oral epithelial cells. *Mol Oral Microbiol*. 2010; 25: 215–225.
15. Allen HL, Deepe GS. Apoptosis modulates protective immunity to the pathogenic fungus *Histoplasma capsulatum*. *J Clin Invest*. 2005; 115: 2875–2885.
16. Chandra J, Zhivotovsky B, Zaitsev S et al. Role of apoptosis in pancreatic beta-cell death in diabetes. *Diabetes*. 2001; 50 (Suppl 1): S44–S47.
17. Reed JC. Bcl-2 family proteins. *Oncogene*. 1998; 17: 3225–3236.
18. Barla F, Higashijima H, Funai S et al. Inhibitive effects of alkyl gallates on hyaluronidase and collagenase. *Biosci Biotechnol Biochem*. 2009; 73: 2335–2337.
19. Andréo R, Regasini LO, Petrônio MS et al. Toxicity and loss of mitochondrial membrane pPotential Induced by aAlkyl gallates in *Trypanosoma cruzi*. *Int Sch Res Notices*. 2015; 2015: 924670.
20. Cacere CR, Romano CC, Mendes Giannini MJ et al. The role of apoptosis in the antigen-specific T cell hyporesponsiveness of paracoccidioidomycosis patients. *Clin Immunol*. 2002; 105: 215–222.