

Original Article

Gliotoxin produced by *Aspergillus fumigatus* induces apoptosis of human bronchial epithelial cells via the Bcl-2 pathway

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Abstract: Fungal secondary metabolite gliotoxin (GT) is the major virulence factor of *Aspergillus fumigatus* (*A. fumigatus*), which causes invasive aspergillosis in immunocompromised individuals. GT has a wide range of effects on metazoan cells in culture, including induction of apoptosis through inhibition of NF- κ B and inhibition of superoxide production by phagocytes. These activities have led to the proposal that GT contributes to invasive aspergillosis pathogenesis. We tested this hypothesis by constructing an *in vitro* model of human bronchial epithelial cells infected by isogenic GT-producing and non-producing strains. Deletion of gliP resulted in B-5233 strains that were wild type for growth, but they did not synthesize GT. We found that both Af293 and B-5233wt strains had greater abilities to induce apoptosis of human bronchial epithelial cells, compared to the B-5233 Δ gliP strain. Furthermore, the expression of the pro-apoptotic Bcl-2 family member Bak significantly increased in the three groups, especially in cells infected with GT-producing strains. These results suggest that there is a positive correlation between production of GT and virulence of *A. fumigatus* and that the pro-apoptotic Bcl-2 family member Bak is pivotal to GT-induced apoptosis.

Keywords: *A. fumigatus*, gliotoxin, Δ gliP, bronchial epithelial cells, Bak

Introduction

Invasive aspergillosis is an opportunistic disease caused by the airborne fungus *A. fumigatus* in humans [1]. In healthy individuals, the inhaled conidia of *A. fumigatus* are cleared by the intraepithelial immune system of the lung. However, in immunocompromised patients, such as organ or allogeneic stem cell transplant recipients, and patients with HIV infection or acute leukemia [2-4], *A. fumigatus* conidia can attach to the lung epithelium, germinate and produce a mass of hyphae, which may cavitate and potentially destruct the surrounding lung parenchyma [5]. Despite advances in early diagnosis and new antifungal agents, invasive aspergillosis remains a leading cause of death in this patient population, with an attributable mortality rate ranging from 30% to 80% [6]. It is therefore crucial to elucidate the molecular mechanisms by which *A. fumigatus* breach the

alveolar epithelial cell barrier. However, the interaction between *A. fumigatus* and the respiratory epithelium has received relatively little attention, compared to specialized cells such as polymorphonuclear neutrophils and macrophages [7]. Most studies showed that some airway cell lines are able to react to fungal components by producing different kinds of cytokines [8-10]. However, *in vitro* experimental systems that were used to study the virulence of *A. fumigatus* toward human bronchial epithelial cells were less developed.

Filamentous fungi produce an array of chemical called secondary metabolites, including antibiotics such as penicillin, HMG-CoA reductase inhibitors such as statins, and toxins such as trypacidin, fumonisin, and GT. These secondary metabolites are toxic to humans and animals and help fungi invade host tissues [11]. GT is a member of the epipolythiodioxopiperazine

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(ETP) family, characterized by an internal disulfide bridge, a diketopiperazine ring and an aromatic amino acid, phenylalanine [12]. In 2004, Gardiner et al discovered a 12-gene cluster in the *A. fumigatus* genome sequence [12], and at the same time, five laboratories embarked on the functional analysis of this gene cluster, in the *A. fumigatus* strains Af293 and CEA10, or the clinical isolate B-5233, by disrupting either the *gliP* gene encoding a nonribosomal peptide synthase (NRPS) or the *gliZ* gene encoding a transcription factor of GT biosynthesis, which contributes to *A. fumigatus* virulence [13-17]. It had been demonstrated that NRPS is responsible for the first step of GT biosynthesis. Although the biological activities of GT are still under investigation, it is known that this molecule has many abilities, including the following: inducing host cell apoptosis [18-20], inhibition of macrophage and polymorphonuclear cell functions; inhibition of NF- κ B, a transcriptional regulator of the host proinflammatory response; and inactivation of enzymes such as alcohol dehydrogenase, creatine kinase, and farnesyl-transferase [19, 21]. In this study, we analyzed the contribution that GT makes to the virulence of *A. fumigatus* and the role of apoptosis in its mechanisms.

Materials and methods

16HBE14o-cell line and reagents

The human bronchial epithelial 16HBE14o-cell line was purchased from the Feilei Company (Shanghai, China). This cell line retains key characteristics of human bronchial epithelium, including the formation of a monolayer, tight junctions, and directional ion transport [22]. HBE14o-cultures were maintained in low-glucose Dulbecco's Modified Eagles Medium (DMEM) containing 10% (v/v) heat-inactivated fetal bovine serum (FBS) at a final concentration of 50 units/ml penicillin G and 50 μ g/mL streptomycin at 37°C in humidified 5% CO₂. Cells were seeded and grown to confluence (5 \times 10⁶ cells) in a 9 cm-dish (Corning Costar, New York, USA) for co-incubation experiments. All cell preparations contained more than 95% viable cells, as judged by trypan blue exclusion. GT (purity: 99%) was purchased from Sigma-Aldrich (Taufkirchen, Germany) and dissolved in dimethyl sulfoxide (DMSO). For apoptosis induction, 1 \times 10⁶ cells/mL were incubated with the indicated concentration of GT diluted in DMEM

for 24 h, and apoptosis assays were performed as described below.

Fungal strains and culture conditions

Three *A. fumigatus* isolates were investigated in this study. *A. fumigatus* strain Af293, used in the sequencing project [3], was kindly provided by CBS-KNAW (Utrecht, Netherlands), and both *A. fumigatus* B-5233wt and B-5233 Δ gliP strains were provided by the Academy of Military Medical Sciences (Beijing, China). These strains were sub-cultured on a potato dextrose agar plate at 30°C for 5 days to ensure sufficient formation of conidia. Mature conidia were harvested by flooding the surface of the plate with 5 mL phosphate buffered saline (PBS) containing 0.025% (v/v) Tween-20. The resulting conidial suspension was vortexed and passed through a sterile gauze in a plugged funnel to eliminate hyphae. The suspension was again vortexed, pelleted, and resuspended in 1 mL PBS. At last, the suspension was diluted to 5 \times 10³ conidia/mL in DMEM by quantification with a hemocytometer for co-incubation experiments.

Construction of 16HBE14o- and A. fumigatus conidia co-culture in vitro

When 16HBE14o-monolayers reached confluence (5 \times 10⁶ cells), the culture dishes were washed in PBS to remove unbound cells. The *A. fumigatus* conidia suspension (MOI, 1 conidium/1000 cells) prepared was added to the dish and co-incubated with 16HBE14o-cells for 2 hours at 37°C in humidified 5% CO₂ to let the fungus adhere to the cell surface. The dishes were washed three times in PBS to remove unbound conidia, supplemented with an equal volume of DMEM, and incubated at 37°C in humidified 5% CO₂ for 12, 24, and 36 hours. The culture supernatant was collected for extraction and for detection of GT by liquid chromatography-mass spectrometer (LC-MS/MS), and 16HBE14o-cells were analyzed for apoptosis by annexin V staining, RT-PCR, and western blot.

Extraction and detection of GT

GT extraction was performed as described before [16]. In brief, the sterile filtered (0.22 μ m) culture supernatant was extracted with an equal volume of chloroform for 30 min at room

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temperature and dried in a rotary evaporator under negative pressure, and finally the metabolite was dissolved in 10-100 μ L methanol.

The presence or absence of GT was determined using LC-MS/MS. As a control for the extraction procedure, a GT standard was run to ensure the identity of the product and to provide an estimation of the amount of GT in the culture medium. One microliter of extract was loaded onto a 4.6 \times 250 mm analytical reversed phase column (ZORBAX Eclipse XDB-C18, Agilent Technologies, California, USA) equilibrated with 0.02% trifluoroacetic acid in 28% acetonitrile/ H_2O . The sample was eluted isocratically following the UV absorbance at 254 nm and the signal on a LCMS-8040 mass spectrometer. Mass spectra were collected in the positive ion mode, with scanning between 261 and 325 m/z. To assay for the presence of GT in the samples, an extracted ion current was calculated that consisted of 3 characteristic positive ions that resulted from partial fragmentation or degradation of the GT molecule (parent ion [327 amu], dethiogliotoxin [263 amu], and further neutral loss of H_2O from dethiogliotoxin [245 amu]). Injection of 1 μ L of a GT standard (Sigma Aldrich) was found to elute 10 min after injection, as indicated by readily apparent peaks in both the UV and extracted ion current chromatograms and characteristic gliotoxin ions.

Analysis of purified GT toxicity on 16HBE14o-cells by CCK-8

The toxicity of purified GT on 16HBE14o-cells cultured with GT for 24 hours was determined by a Cell Counting Kit-8 assay (Beyotime, Shanghai, China). At first, GT (purity: 99%) was purchased from Sigma-Aldrich (Taufkirchen, Germany) and dissolved in dimethyl sulfoxide (DMSO). Second, 1×10^4 cells/mL (100 μ L) were inoculated to the 96-well culture plate and incubated with the indicated concentration of GT for 24 h at 37°C in humidified 5% CO_2 . At last, 10 μ L CCK-8 working liquid were joined to the 96-well culture plate and incubated for 1 h at 37°C in humidified 5% CO_2 . OD value was determined at 450 nm wavelength.

Analysis of 16HBE14o- apoptosis by annexin V staining

The percentage of the apoptotic 16HBE14o-cells in different experiments was determined by analyzing phosphatidylserine exposure and

membrane integrity by double-staining with FITC-conjugated anti-annexin V antibody and propidium iodide (PI) (Keygentec, China), and flow cytometric analysis (Cell Quest Pro Software, Becton-Dickinson, Franklin Lakes, USA), as follows: Cells (10^4) were washed with PBS, resuspended in 50 μ L of annexin binding buffer (Becton-Dickinson Biosciences, New Jersey, USA), and incubated for 10 min at room temperature in the dark with an FITC-conjugated anti-annexin V antibody and PI (final concentration of 1 μ g/mL). The cells were washed and resuspended in binding buffer, and flow cytometric analysis was performed within 30 min of staining. The extent of apoptosis observed was reported as a percentage using the following formula: apoptotic fraction = (annexin V + PI events) / [(annexin V + PI events) + (annexin V - PI events)].

Determination of 16HBE14o-RNA

Total RNA was isolated from 16HBE14o-cells using TRIzol Reagent (Invitrogen, California, USA). After addition of 1 mL TRIzol Reagent, 1.5×10^6 cells were homogenized and incubated at 15-30°C for 30 min to permit complete dissociation of nucleoprotein complexes. After addition of 0.2 mL chloroform per 1 mL TRIzol reagent, tubes were shaken vigorously by hand for 15 s, incubated at 15-30°C for 15 min, and centrifuged at 12,000 g for 15 min at 4°C. RNA in the aqueous phase was transferred to a clean tube, and 0.5 mL isopropyl alcohol was added per milliliter of TRIzol reagent. The mixture was incubated at 15-30°C for 15 min and centrifuged at 12,000 g for 15 min at 4°C. After removing the supernatant, the RNA pellets were washed once with at least 1 mL 75% ethanol, mixed by vortexing, and centrifuged at 12,000 g for 5 min at 4°C. After removing the supernatant, the RNA pellets were dried briefly, and 30-50 μ L RNase-free water was added for quantification. RNA yield from each population was determined using Tecan infinite 200 (Switzerland). RNA yields ranged from 30 to 100 ng/ μ L. RNA integrity of the sorted samples were determined using an electrophoresis method.

RT-PCR and western blot analysis for the pro- and anti-apoptotic Bcl-2 family members

One microliter of total RNA (1 ng/ μ L) was reverse transcribed using the TaKaRa RNA Transcription Kits (Osaka, Japan). RT-PCR was

Table 1. Name and sequence of primers used in these studies

Primers	Upstream sequence (5'→3')	Downstream sequence (5'→3')
Bcl-2	TGCGACCTGATTCATTG	TGGTTATCGTACCCTGTTCTC
Bak	TGAGTCATAGCGTCGGTTG	GCACAGGGACAAGTAAAGGC
Bax	GGACCCGGTGCCTCAGGA	CAAAGATGGTCACGGTCTGC
GAPDH	TGGGGTGATGCTGGTGCTGAGT	AGGTTTCTCCAGGCGGCATGTC

performed in 96-well plates using 10 µl SYBR Premix Ex Taq, 1 µL of each forward and reverse primer for the candidate gene (10 µM stock), 7 µL of distilled H₂O and 1 µL of the reverse-transcribe cDNA per well. As a negative control, H₂O was used instead of SYBR Premix Ex Taq, and for normalization, the housekeeping gene GAPDH was co-amplified. The plate was sealed with adhesive foil, spun down at 1000 rpm for 1 min, and subjected to PCR in an ABI 7500 apparatus (Applied Biosystems, California, USA). For each cDNA sample, triplicates were measured with the two primer pairs (gene of interest and housekeeping gene). The primers are given in **Table 1**.

Western blot analysis of pro- and anti-apoptotic Bcl-2 pathway proteins was performed as described previously [23, 24]. 16HBE14o-cells collected at different time points were washed with ice-cold PBS and added to phenylmethanesulfonyl fluoride (PMSF) purchased from Amresco (Solon, USA). The cells were lysed in RIPA Lysis Buffer (50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 1% NP40) purchased from Beyotime Biotechnology Company (Shanghai, China) and cleared by centrifugation at 10,000 g for 5 min at 4°C. For Western blot analysis, equal quantities of protein were run on 12% acrylamide gels and transferred onto nitrocellulose membranes. After blocking with 5% nonfat dried milk in Tris buffered saline, the membrane was incubated with the desired primary antibody overnight at 4°C. The membrane was then treated with appropriate peroxidase-conjugated secondary antibody and bands were visualized using an enhanced chemiluminescence method. Each membrane was stripped and probed with an antibody against actin to correct for differences in protein loading. The following antibodies were used: anti-Bcl-2 (Abcam, London, Britain), anti-Bak (CST, Boston, USA), and anti-Bax (CST, Boston, USA). Horseradish peroxidase-labeled anti-rabbit antibody and anti-rat antibody were used as secondary antibodies. Quantitation of the immuno-

reactive bands was performed using a chemiluminescence imaging system (Bioshine, Shanghai, China). Immunoblotting was performed at least twice using independently prepared lysates to ensure reproducibility of the results.

Statistical analysis

Data were expressed as the mean ± standard deviation. Statistical analysis was performed by SPSS for Windows. Student's t-test was used to compare the statistical significance of the difference in data from the two groups; *P* < 0.05 was considered statistically significant. All experiments were performed three times.

Results

Production of GT by A. fumigatus in co-cultures

To study the relationship between the synthesis of GT and the virulence of *A. fumigatus* isolates under conditions similar to those for human invasive aspergillosis. After 16HBE-14o and three *A. fumigatus* isolates, that is, *A. fumigatus* Af293 strain, *A. fumigatus* B-5233wt and B-5233ΔgliP strains co-culture *in vitro* were constructed for different time, the GT content of the culture supernatant was investigated by LC-MS/MS. We found that GT was readily detected for the *A. fumigatus* Af293 and B-5233wt strains and was absent in the *A. fumigatus* B-5233wt ΔgliP strain (**Figure 1A**). These results verified that the construction of the *A. fumigatus* B-5233wt ΔgliP strain was successful. Furthermore, to study whether 16HBE-14o could stimulate the production of GT in co-culture, Af293 strain was cultured without 16HBE-14o *in vitro* for different time, and the GT content of the culture supernatants was investigated by LC-MS/MS. We found that the production of GT in the group with 16HBE-14o was more than that in the group without 16HBE-14o, especially at 36 h (**Figure 1B**).

GT is the important virulence factor of A. fumigatus in co-cultures

To evaluate the putative role of GT in the virulence of *A. fumigatus*, 16HBE14o-cells co-cultured with three *A. fumigatus* isolates were collected at different time points and subsequently stained with Annexin V and PI. As a

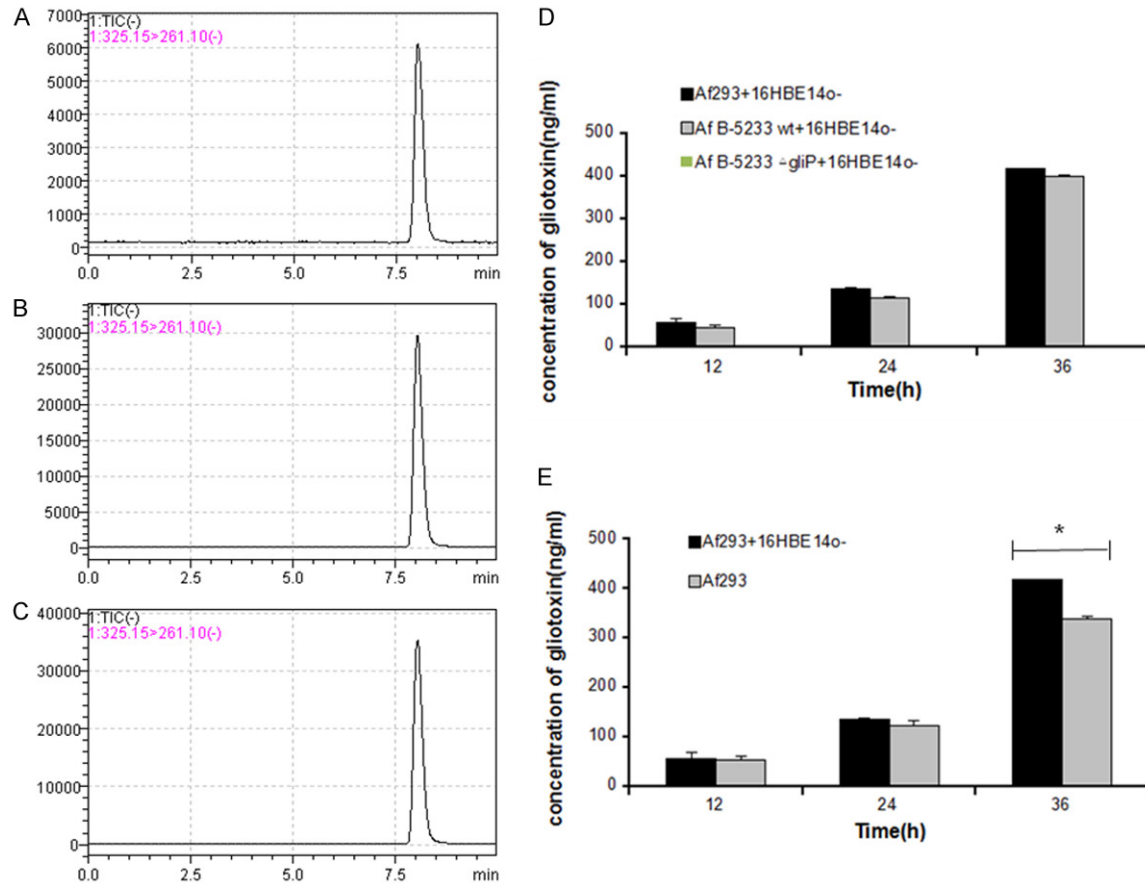


Figure 1. Liquid Chromatography-Mass Spectrometer/Mass Spectrometer analysis for GT production at 28 nm of gradient elution of samples from culture supernatant are shown. A. Chromatogram of gliotoxin eluted after 10 min as determined with a purified gliotoxin standard (1 µg/ml); B. Chromatogram of gliotoxin extracted from the supernatant was generated when Af293 strain was cultured without 16HBE-14o in vitro for 36 hours; C. Chromatogram of gliotoxin extracted from the supernatant was generated when Af293 strain was cultured with 16HBE-14o in vitro for 36 hours; D. GT was readily detected for the *A. fumigatus* Af293 and B-5233wt strains and was absent in the *A. fumigatus* B-5233wt ΔgliP strain. After 16HBE14o- and three *A. fumigatus* isolates co-culture in vitro were constructed for the different time periods; E. The production of GT in the group with 16HBE14o- was more than that in the group without 16HBE14o-, the differences between the two groups were statistically significant ($P < 0.05$) at the 36 h time-point.

negative control, 16HBE14o-cells were separately cultured in DMEM. For the three *A. fumigatus* isolates, significant proportions of the cells (51.75% for Af293, 52.92% for *A. fumigatus* B-5233wt, and 36.56% for *A. fumigatus* B-5233wt ΔgliP) were in the late apoptotic stage after 36 h of co-culture, compared to 5.12% for the untreated control cells ($P < 0.01$). These results proved that *A. fumigatus* can induce apoptosis of the 16HBE14o-cells whether *A. fumigatus* secretes GT or not. However, the apoptotic cell percentages induced by Af293, and B-5233wt (51.75 and 52.92%, respectively) were significantly higher than that induced by the B-5233wt ΔgliP strain (36.56%)

at the 36h time-point ($P < 0.05$). The virulence of *A. fumigatus* was obviously weakened by the lack of GT; therefore, GT may play an important role in invasive aspergillosis (Figure 2).

GT causes apoptosis of 16HBE14o-cells via the Bcl-2 pathway

It was previously reported that GT induces apoptosis in mouse embryonic fibroblasts in a caspase- and Bak-dependent manner [20]. As GT primarily targets the lung epithelium *in vivo*, we tested whether the toxin killed the human bronchial epithelial 16HBE14o-cells by the same mechanisms *in vitro*. Total RNAs of the

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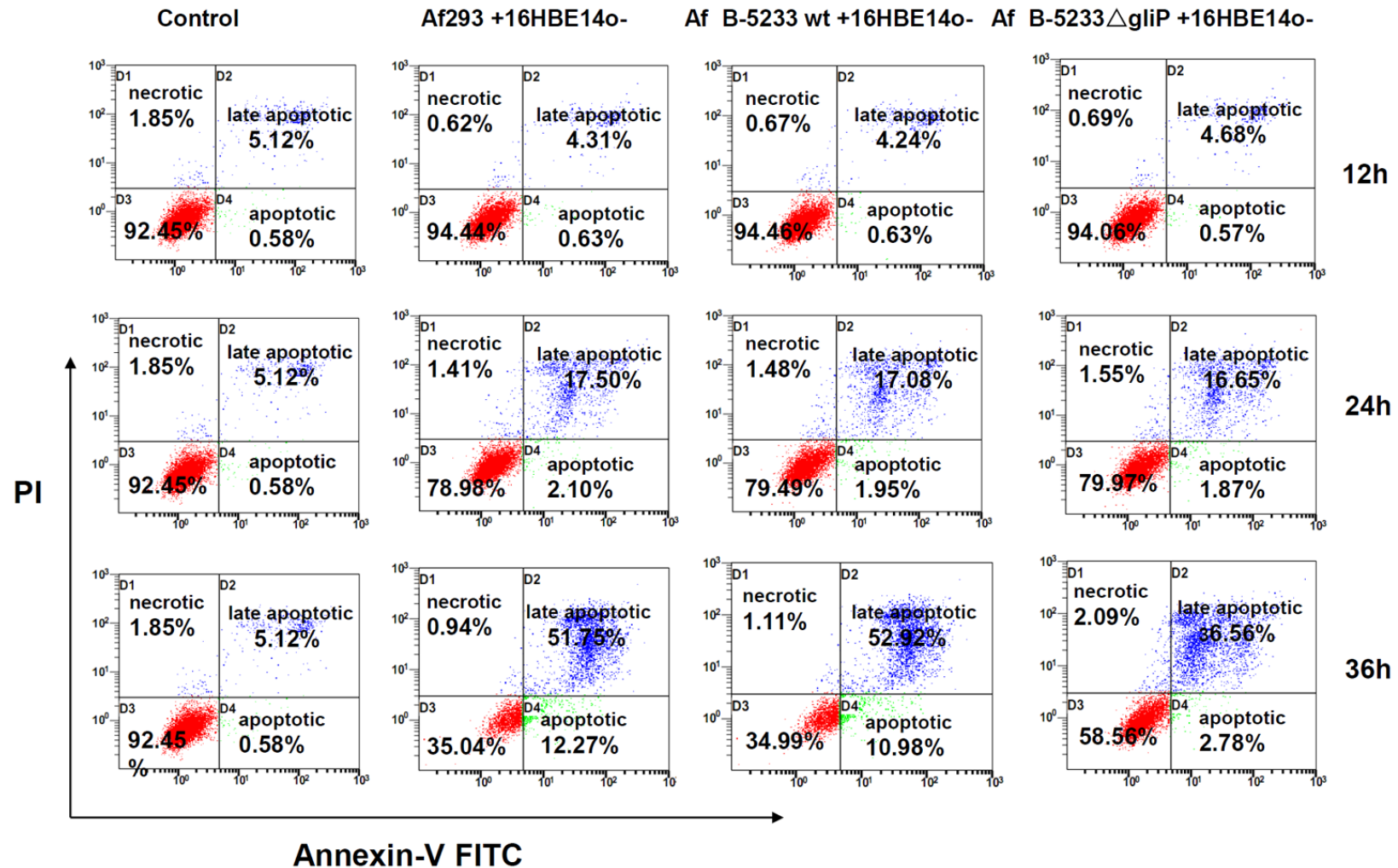


Figure 2. Flow cytometry analysis of cells apoptosis in the in vitro model of human bronchial epithelial cells infected by *A. fumigatus* for the different time. Cells were collected and double stained with FITC-conjugated Annexin V and propidium iodide; the fluorescence was quantified by FACS analysis. A significant proportion of the cells (17.50% for Af293, 17.08% for Af B5233 WT and 16.65% for Af B5233ΔgliP, respectively) were even in the late apoptotic stage after 24 h of incubation ($P < 0.05$). Repeated experiments showed similar results. The differences between the former two groups (Af293 and Af B5233 WT) and the last group (Af B5233ΔgliP) were statistically significant ($P < 0.005$). The showed results are the mean \pm SD of triplicate determinations; statistical significance of results was evaluated by student's t-test. * $P < 0.05$; ** $P < 0.01$; and *** $P < 0.005$. The experiments were repeated twice.

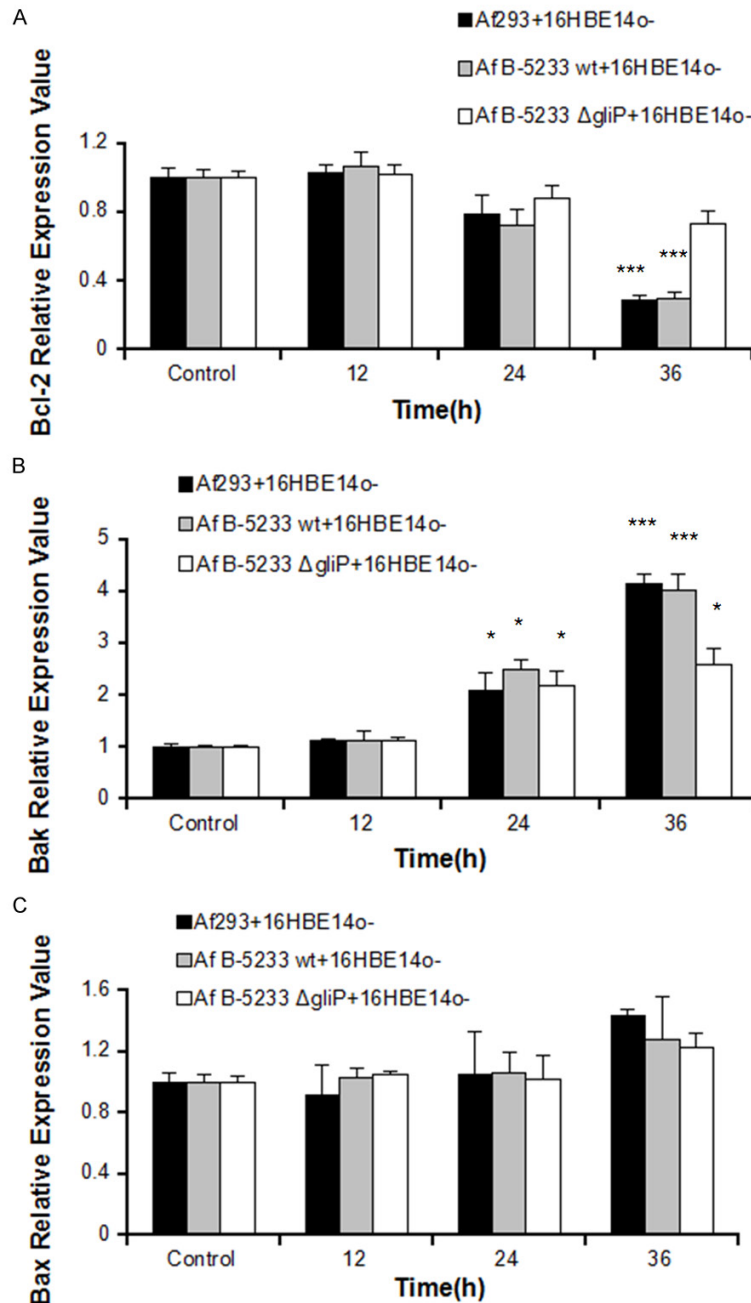


Figure 3. 16HBE14o- was incubated with three strains (Af293, Af B5233 WT and Af B5233ΔgliP) for indicated time periods. The pro-apoptotic Bcl-2 family member Bcl-2, Bax and Bak was determined by RT-PCR as described in the Methods section. Compared to the group of Af B5233ΔgliP, obvious changes in the Bak and Bcl-2 were observed for Af293 and Af B5233 WT over the experimental period, especially at 36 h ($P < 0.005$). Furthermore, the differences for the Bak between Af B5233ΔgliP at 36 h and control were still statistically significant ($P < 0.05$).

cells co-cultured respectively with the three *A. fumigatus* isolates were extracted, and the expression of the pro- and anti-apoptotic Bcl-2 family members (Bcl-2, Bax, and Bak) was detected by RT-PCR. For *A. fumigatus* Af293 and B-5233wt strains producing GT, the expres-

sion of the Bcl-2 and Bak genes was significantly lower and higher, respectively, than that of the control ($P < 0.005$) at the 36 h time-point. For the *A. fumigatus* B-5233wt ΔgliP strain that does not produce GT, the expression of the Bak gene was slightly higher, although significant, than that of the control ($P < 0.05$), but the Bcl-2 gene expression did not change ($P > 0.05$), at the 36 h time-point. Therefore, the expression level of the pro-apoptotic Bcl-2 family member Bak was significantly different in each three groups after 36 hours (Figure 3). In a word, the pro-apoptotic Bak and anti-apoptotic Bcl-2 are part of an apoptosis pathway that may be important in the apoptosis of 16HBE14o- induced by GT. These results were confirmed by western blot analysis (Figure 4).

The expression of the pro-apoptotic Bcl-2 family member Bak is increased directly by GT-induced apoptosis

From the above experiments, we detected apoptosis of human bronchial epithelial cells and expression of the pro-apoptotic Bcl-2 family member Bak fairly consistently between *A. fumigatus* strains producing GT and an *A. fumigatus* strain not producing GT. Therefore, we wished to determine whether purified GT shows the same virulence for human bronchial epithelial cells *in vitro*. At first, we analyzed the toxicity of purified GT on 16HBE14o-

cells cultured with GT for 24 hours in a CCK-8 assay. The results showed that GT had obvious toxic effects on 16HBE14o- cells; with increases in GT concentration, the toxicity increased gradually. The concentration of GT that caused a 50% reduction in cell viability was 0.25 μg/mL (Figure 5). To further dif-

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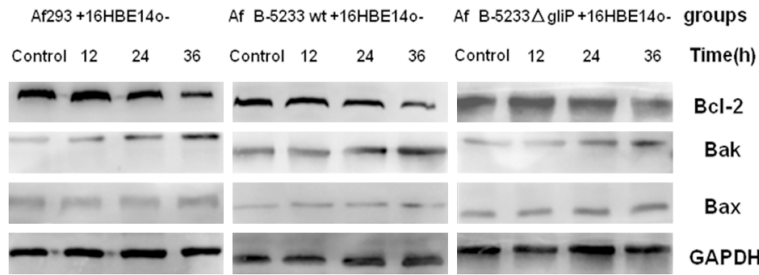


Figure 4. The pro-apoptotic Bcl-2 family member Bcl-2, Bax and Bak was determined by western blot as described in the Methods section. The results showed that the successful downregulation of Bak at the group of Af B5233ΔgliP, Compared to the another two groups. GAPD immunoblotting served as control for equal protein loading.

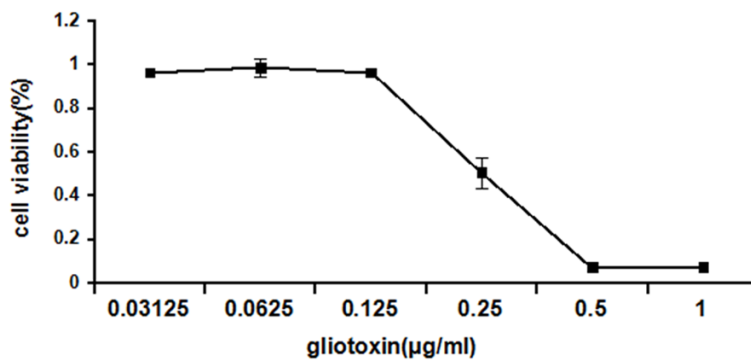


Figure 5. Cell viability was determined via CCK-8 assay at specified time-point, after 16HBE14o- were washed and incubated in DMEM medium with indicated concentrations of GT. The results showed that GT had obvious toxic effects to 16HBE14o-. With the increase of GT concentration, the toxicity increased gradually. GT (0.25 μg/ml) could cause 50% reduction in viability at 1 h.

ferentiate between induction of apoptosis and necrosis, 16HBE14o-cells cultured with GT were collected and subsequently stained with Annexin V and PI. Although necrotic cells were observed, the percentage of apoptotic cells significantly increased ($P < 0.05$) with increase in GT concentration (36.79% for 0.25 μg/mL GT and 52.65% for 1.0 μg/mL GT) (**Figure 6**). Thus, GT did induce apoptosis of 16HBE14o-*in vitro*.

We next studied whether the pro-apoptotic Bcl-2 family member Bak played an important role in GT-induced apoptosis, by detecting the expression of the pro- and anti-apoptotic Bcl-2 family member by RT-PCR. The results showed that the expression of the pro-apoptotic Bcl-2 family member Bak and Bax were increased, especially Bak expression (**Figure 7**). Therefore, purified GT toxicity induces the apoptosis of 16HBE-14o cells *in vitro* and may be closely

related to the expression of the pro-apoptotic Bcl-2 family member Bak.

Discussion

GT is considered to be an important virulence factor that affects the course of invasive aspergillosis. While many studies show the toxic and immunosuppressive effect of this mycotoxin in specialized cells such as polymorphonuclear neutrophils, macrophages and lymphocytes [7, 17, 19, 20], the number of detailed studies for its role in the interaction between *A. fumigatus* and the respiratory epithelium is limited. Therefore, the *in vitro* model of human bronchial epithelial cells infected by *A. fumigatus* is constructed to further explore the virulence of *A. fumigatus*. In the present study, 16HBE14o-cell monolayers seeded in 9 cm-dish were grown to confluence (5×10^6 cells), and the prepared *A. fumigatus* conidia suspension (MOI 1 conidia/1000 cells) was added to the mono-

layer. At last, we checked the relevant data at different time points (12 h, 24 h and 36 h) and observed the dynamic development on the virulence of *A. fumigatus* as the extension of the incubation time. In this process, the fungal spores became hyphae, while epithelial cells devoured the fungal spores at first and killed by hyphae at last. Thus, our results could be true and close to invasive aspergillosis.

GT production was determined predominantly for *A. fumigatus*, and the secretion levels depended on the culture conditions. In our study, we checked the GT content of the culture supernatant at first, these results indicated that the concentration of GT increased obviously as the extension of incubation time, for example, its concentration could reach 420 ng/mL at the 36 h time-point, which was far higher than that caused a 50% reduction in cell

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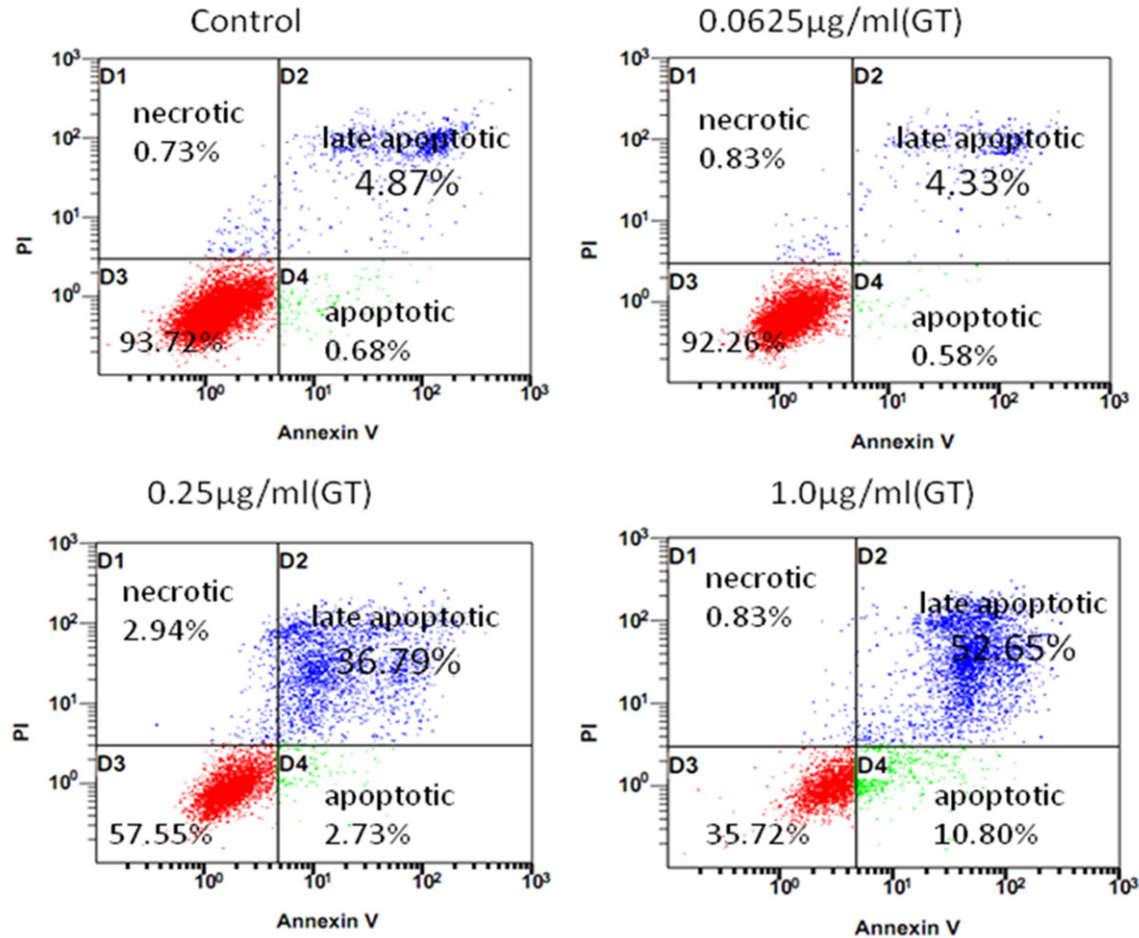


Figure 6. 16HBE14o were incubated with 0.0625 µg/ml, 0.25 µg/ml and 1.0 µg/ml GT for 1 h, then collected and double stained with FITC-conjugated Annexin V and propidium iodide, the fluorescence was quantified by FACS analysis. The results showed that the apoptosis rate of cells had a significantly rising, with the increase of GT concentration (36.79% for 0.25 µg/ml GT and 52.65% for 1.0 µg/ml GT). The differences for the apoptosis rate of cells between all experiments groups and control were statistically significant ($P < 0.05$). The show results are the mean \pm SD of triplicate determinations; statistical significance of results was evaluated by student's t-test. The experiments were repeated twice.

viability (250 ng/mL). Some studies also demonstrated that the most abundant mycotoxin produced by *A. fumigatus* is GT [1]. Therefore, an explanation for GT as an important factor on the virulence of *A. fumigatus*, is large amount of secretion. Furthermore, we also found that the concentration of secreted GT in the group with human bronchial epithelial cells was higher than that in the group without human bronchial epithelial cells, especially at the 36 h time point, which indicated that human bronchial epithelial cells could stimulate the secretion of GT. However, there is no clear explanation at the present. Some studies showed that human bronchial epithelial cells produced different kinds of cytokines [8-10] and swallow fungal

spore, which could stimulate the defensive system of *A. fumigatus*.

The condensation of serine and phenylalanine catalyzed by the NRPS gliP is the first step in GT biosynthesis, so the B-5233ΔgliP strain used in our study were confirmed not to produce GT by LC-MS/MS. Previous studies showed GT could induce relatively specific and rapid apoptosis of monocytes and monocyte-derived dendritic cells (DC), resulting in impaired presentation of target antigens to effector T cells and the attendant limitation of an efficient adaptive immune response [25]. At the present, our study showed that gliP deletion mutants had significantly reduced virulence

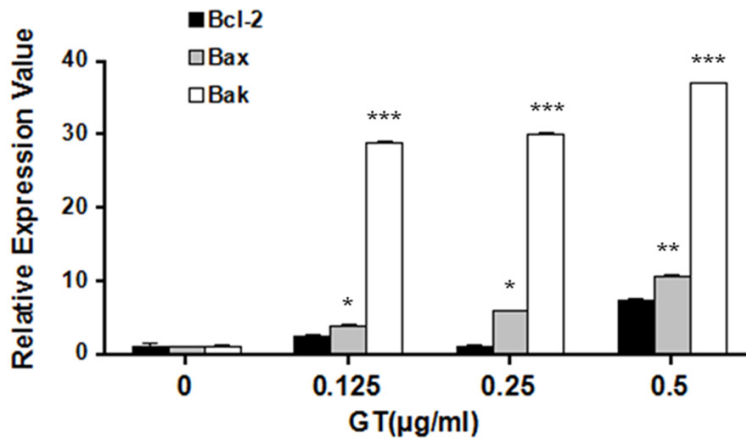


Figure 7. RT-PCR analysis for the expression of the pro- and anti-apoptotic Bcl-2 family member, when 16HBE14o were incubated with different concentrations GT for 1 h. The results showed that the expression of the pro-apoptotic Bcl-2 family member Bak and Bax were increased, especially Bak expression. Compared to the control, there were obviously statistical significance at the different concentrations of GT ($P < 0.005$). The show results are the mean \pm SD of triplicate determinations; statistical significance of results was evaluated by student's t-test. * $P < 0.05$; ** $P < 0.01$; and *** $P < 0.005$. The experiments were repeated twice.

to human bronchial epithelial cells, compared to the wild-type parental strain and Af293 strain. The apoptosis rate of the front group was more or less half of the two later groups at the 36 h time-point, which demonstrated that GT contributes to the important role in the virulence of *A. fumigatus*. Furthermore, for GT, inducing the apoptosis of numerous cells was an important tool.

In addition, we checked the expression of the pro- and anti-apoptotic Bcl-2 family members by RT-PCR and western blot. Our results showed that the expression of the pro-apoptotic Bcl-2 family member Bak was directly related to the production of GT and the apoptosis rate of 16HBE14o- previous report found that 5 out of 6 wild-type mice succumbed within 2 weeks after intranasal infection; however, only 1 out of 6 *Bak*^{-/-} mice succumbed over the same time period, probably because Bak is directly activated by GT [20]. GT, an ETP class of toxins produced by *A. fumigatus*, is known to cause apoptosis in professional immune cells such as macrophages and monocytes [25, 26], as well as in non-immune cells, by directly activating the pro-apoptotic Bcl-2 family member Bak, as reported by Pardo et al [20].

Some studies have demonstrated that the Bcl-2 family is an important set of proteins

involved in the regulation of apoptosis [27, 28]. These proteins can be broadly divided into three classes: (1) anti-apoptotic members such as Bcl-2 and Bcl-X_L with sequence homology at BH (Bcl-2 homology)1, BH2, BH3, and BH4 domains; (2) pro-apoptotic molecules, such as Bax and Bak, with sequence homology at BH1, BH2 and BH3; and (3) pro-apoptotic proteins that share homology only at the BH3 domain, such as Bid, Bik, Noxa, and Bim [27, 29]. Over the past decade, intensive biochemical studies have been performed on the Bcl-2 family, particularly concerning the relationships between the Bcl-2 family and cytochrome C. Pro- and anti-apoptotic Bcl-2 proteins regulate apoptosis

in part by controlling cytochrome C release from mitochondria.

In conclusion, we have demonstrated in our study that GT played an important role in inducing the apoptosis of 16HBE14o-cells by *A. fumigatus* and by activating the pro-apoptotic Bcl-2 family member Bak pathway. Further experiments using this *in vitro* model should be performed to explore the virulence of *A. fumigatus* in more details. For example, the secretome of human bronchial epithelial cells in response to *A. fumigatus*, which has been analyzed using differential in-gel electrophoresis [23], as well as transcriptome analysis of *A. fumigatus* and human bronchial epithelial cells, is being studied to determine the mechanisms at play in the induction of human bronchial epithelial cell apoptosis by *A. fumigatus*.

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Disclosure of conflict of interest

None.

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