1   **Research article**

2   **The Inhibitory Effect of Validamycin A on *Aspergillus flavus***

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Abstract

Aspergillus flavus is one of the most common isolates from patients with fungal infections. *Aspergillus* infection is usually treated with antifungal agents, but side effects of these agents are common. Trehalase is an essential enzyme involved in fungal metabolism and the trehalase inhibitor, validamycin A, has been used to prevent fungal infections in agricultural products. In this study, we observed that validamycin A significantly increased trehalose levels in *A. flavus* conidia and delayed germination, including decreased fungal adherence. In addition, validamycin A and amphotericin B showed a combinatorial effect on *A. flavus* ATCC204304 and clinical isolates with high minimum inhibitory concentrations (MICs) of amphotericin B using checkerboard assays. We observed that validamycin A and amphotericin B had a synergistic effect on *A. flavus* strains resistant to amphotericin B. The MICs in the combination of validamycin A and amphotericin B were at 0.125 μg/mL and 2 μg/mL, respectively. The FICI of validamycin A and amphotericin B of these clinical isolates was about 0.25-0.28 with synergistic effects. No drug cytotoxicity was observed in human bronchial epithelial cells treated with validamycin A using LDH-cytotoxicity assays. In conclusion, this study demonstrated that validamycin A inhibited the growth of *A. flavus* and delayed conidial germination. Furthermore, the combined effect of validamycin A with amphotericin B increased *A. flavus* killing, without significant cytotoxicity on human bronchial epithelial cells. We propose that validamycin A could potentially be used *in vivo* as an alternative treatment for *A. flavus* infections.

Introduction

Aspergillus flavus is a fungus commonly found in the environment and when it contaminates food, it produces aflatoxins which are associated with increased risk of developing liver cancer in humans [1, 2]. Moreover, *A. flavus* is an infectious fungus and can colonise organs leading to conditions such as keratitis, cutaneous infections, sinusitis, and invasive pulmonary aspergillosis [3-5]. Knowledge and understanding of the epidemiology and pathogenesis of *A. flavus* infection in humans is still very limited as there are only a few reports on *A. flavus* in comparison to other *Aspergillus* species [6]. For example, it has been reported that *A. flavus* is a common cause of cutaneous infections and sinusitis in India [4, 5].

Initial treatment of *Aspergillus* invasive infections (invasive aspergillosis) begins with antifungal agents, particularly azoles. Voriconazole is a drug of choice in patients with aspergillosis [7, 8] but serious adverse reactions have been reported in many studies, such as transient visual disturbances, hepatotoxicity, tachyarrhythmias, and QTc interval prolongations [8]. Amphotericin B is a fungicidal polyene agent, which is an alternative, relatively cheap treatment for aspergillosis [7, 8] but it also has serious side effects (e.g. nephrotoxicity) [9]. Owing to socioeconomic status of patients and availability of this agent, the use of amphotericin B as a treatment against aspergillosis is very common in developing countries, including Thailand [10-12]. Unfortunately, recent studies have demonstrated increasing incidence of *A. flavus* clinical isolates with resistance to amphotericin B [13, 14].

Although patients with aspergillosis are treated with standard antifungal therapy as mentioned, evidence shows that the morbidity and mortality rates in patients with these infections are still high (up to 80%) [15]. Therefore, the discovery of novel antifungal agents with fewer side effects is crucial for treatment of aspergillosis. Many studies have reported virulence factors and metabolic pathways that are specific to this fungus, and these could potentially be new targets for the development of antifungal agents [16, 17]. For example,

trehalose is a disaccharide that is only found in bacteria, plants, insects, and invertebrates. It is composed of two glucose molecules conjugated with α, α-1, 1-glycosidic linkage and serves as an energy source, particularly when fungi are exposed to environmental stresses such as cold, heat and desiccation [18-20].

There are three different enzymes involved in trehalose pathway; a) trehalose-6-phosphate synthase (Tps1p), b) trehalose-6-phosphate phosphatase (Tps2p) and c) trehalase (Figure 1). Tps1p converts UDP-glucose and glucose 6-phosphate into trehalose 6-phosphate [20]. Tps2p enzyme removes phosphate from trehalose 6-phosphate to form trehalose. These enzymes in the trehalose pathway are essential for the growth of *Candida albicans*, *Cryptococcus neoformans*, and *Aspergillus fumigatus* [18, 21-23]. Trehalase hydrolyzes and degrades trehalose into two glucose molecules [24]. There are two types of trehalase found in *Saccharomyces cerevisiae* [25], which are neutral trehalase and acid trehalase (Figure 2). Neutral trehalase (Nth1p) is found in the cytosol and works at an optimum pH of 7.0 [24, 26] whereas acid trehalase (Ath1p) is a cell wall-linked enzyme and works at an optimum pH of 5.0 [27-29]. It has been reported that the trehalose pathway is involved in the pathogenesis of fungal infections in human (e.g. *C. albicans, C. neoformans, A. fumigatus)* [19, 21-23, 30-32].

In previous studies, it was demonstrated that *Rhizoctonia solani,* a rice fungal pathogen, was inhibited by the trehalase inhibitor, validamycin A [33-35]. Validamycin A was originally isolated from *Streptomyces hygroscopicus* var. *limoneus*, [33, 36, 37] and it was shown that it inhibited branching of *R. solani* [33, 38]. Another study found that validamycin A delayed conidial production of *Fusarium culmorum* [38]. However, the effectiveness of validamycin A against human fungal pathogens and its toxicity on human cells are unknown. Here, we investigated the effects of validamycin A alone and in combination with amphotericin B on the growth of *A. flavus*, including the cytotoxicity of validamycin A to a human cell line.

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Materials and Methods

Fungal strains, media, and conditions

A. flavus ATCC 204304 was cultured on Sabouraud Dextrose Agar (SDA, Oxoid, Thermo Fisher Scientific) petri-dish plates at 37°C for three days before harvesting *A. flavus* conidia using sterile distilled water with 0.01% tween 80. Briefly, 5 mL of sterile distilled water with 0.01% tween 80 was utilized to harvest *A. flavus* conidia on SDA petri-dish plates using cell scrapers. The mixture between distilled water with tween 80 and *A. flavus* conidia was filtered using miracloth. A number of conidia were counted from the filtrate using a hemocytometer. Then 10^3 conidia were inoculated into culture media [39], i.e. glucose peptone agar (peptone 10 g, glucose 20 g, agar 20 g, distilled water 1000 ml, pH 6.8–7.0), trehalose peptone agar (peptone 10 g, trehalose 10 g, agar 20 g, distilled water 1000 ml, pH 6.8–7.0), and peptone agar (peptone 10 g, agar 20 g, distilled water 1000 ml, pH 6.8–7.0), incubated at 37°C for 2-5 days. The radial fungal growth was measured in three biological replicates.

A. flavus clinical isolates were obtained from the Mycology laboratory, Department of Microbiology, Faculty of Medicine, Chulalongkorn University and King Chulalongkorn Memorial Hospital during 2019. Patient characteristics were collected from medical records/charts. Patients with invasive aspergillosis (IA) were classified as proven, probable, and possible invasive aspergillosis, according to EORTC/MSG criteria [40, 41].

Trehalose measurements

Conidia of *A. flavus* ATCC 204304 from SDA treated with or without 1 μg/mL validamycin A were collected at day 5 after incubation at 37°C. Trehalose levels of *A. flavus* conidia were measured, as previously described [42]. Briefly, 2×10^8 conidia in 500 uL distilled water with

120   tween 80 were boiled at 100ºC for 20 min and centrifuged at 11,000xg for 10 min. The

121   supernatant was collected for trehalose measurement (with biological triplicates) using the

122   glucose oxidase assay protocol (Sigma; GAGO20). The reaction was measured at 490 nm using

123   a spectrophotometer (Lambda 1050+ UV/Vis/NIR, PerkinElmer, USA).

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125   **Germination assay**

126   Conidia of *A. flavus* ATCC 204304 at 1×10^8 cells were incubated in 10 mL Sabouraud dextrose

127   broth at 37°C in an orbital shaker at 200 rpm. The cultured broth (500 µL) was used for

128   counting percentage of germlings. The germinated conidia are counted using a microscope. At

129   each time point, 100 conidia were counted and the number of germinated conidia were

130   calculated as a percentage out of total 100 conidia [43]. Each strain was cultured up to 24 h at

131   37°C in three biological replicates [44].

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133   **XTT assay**

134   XTT assays (sodium 2,3 -bis (2-methoxy-4-nitro-5-sulfophenyl) -5- [(phenylamino) -carbonyl]

135   -2H-tetrazolium) were performed as described previously [45, 46]. Briefly, 10^3 conidia of *A.*

136   *flavus* ATCC 204304 were incubated with different culture media with or without validamycin

137   A in 96-well plate at 37°C for 18 h. XTT solution (0.5 mg/mL in PBS) was added into each

138   well, and incubated at 37°C for 15 min. The plate was centrifuged and the supernatant was

139   collected to measure the OD at 490 nm using a spectrophotometer (Lambda 1050+

140   UV/Vis/NIR, PerkinElmer, USA).

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Crystal violet adherence assay

10^5 conidia per mL of *A. flavus* ATCC204304 were incubated in 100 µL of Sabouraud dextrose broth in each well of plastic U-bottomed 96-well plates at 37°C for 24 h. After washing each well twice with sterile distilled water gently, 0.1% crystal violet were utilized to stain for 10 min. Sterile distilled water was then utilized to wash twice and 100% ethanol was used to destain for 10 min. Supernatants were then measured at 600 nm using a spectrophotometer (Lambda 1050+ UV/Vis/NIR, PerkinElmer, USA) [47].

Broth microdilution assay and checkerboard assay

The CLSI broth microdilution M38 method was performed to observe the minimum inhibitory concentrations (MICs) of amphotericin B for *A. flavus* ATCC 204304 and clinical isolates [48]. The additive/synergistic effect of validamycin A and amphotericin B were identified using the checkerboard assays [49]. Fractional inhibitory concentration index (FICI) was calculated for each antifungal drug, in each combination used, with the following formula [49]:

$$\text{FIC A } (MIC_A/MIC_{A+B}) + \text{FIC B } (MIC_B/MIC_{A+B}) = \text{FICI}$$

FICI results were determined as synergy: <0.5; additivity: 0.5-1; indifference: >1-4; and antagonism: >4.

Cell line and culture

BEAS-2B (Human bronchial epithelial cell line) (ATCC® CRL9609™) was cultured with Bronchial Epithelial Cell Growth Basal Medium (BEBM) in tissue culture flasks coated with 0.01 mg/mL fibronectin, 0.03 mg/mL bovine collagen type I, and 0.01 mg/mL bovine serum

164 albumin (BSA). The cells were incubated at 37ºC in a humidified environment with 5% CO_2

165 [50].

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Cytotoxicity assay

168 The cytotoxicity of validamycin A towards human epithelial cell lines was performed using a

169 Lactate Dehydrogenase (LDH)-Cytotoxicity Colorimetric Assay Kit II (Biovision Inc, CA,

170 USA). Briefly, 1 x 10^4 BEAS-2B cells were incubated with 50 µL of DMEM in a pre-coated

171 96-well plate and then validamycin A was added at different concentrations (1µg/mL -

172 1mg/mL, final concentration). LDH reaction mixture was added and the cells were incubated

173 at 37°C for 30 min. LDH released from the cells was measured at 450 nm using a

174 spectrophotometer. The percentage of cytotoxicity was calculated using the following formula:

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$$\text{Cytotoxicity (\%)} = \frac{(\text{test sample - low control}) \times 100}{(\text{high control - low control})}$$

176 High control is cells with lysis buffer while low control is cells alone as a background.

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Statistical analysis

179 All statistical analyses were conducted with Prism 8 software (GraphPad Software, Inc., San

180 Diego, CA). Comparison between groups performed with unpaired two-tailed Student's t-tests

181 for two data groups and one-way ANOVA tests with post-hoc Bonferroni's multiple

182 comparisons tests for more than two data groups. Error bars represent standard errors of the

183 means. Significant differences were considered when p-value < 0.05.

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Ethics statement

This study was approved by the Institutional Review Board (IRB No. 546/60), Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand.

Results

Trehalase homologs in *Aspergillus flavus*

To identify trehalase enzyme homologs in *A. flavus*, a BLASTp search was performed on *S. cerevisiae* and *A. fumigatus* and compared with *A. flavus*. The protein data from FungiDB database and Simple Modular Architecture Research Tool (SMART) were used to compare putative protein domains among trehalase enzymes from *S. cerevisiae* (*Sc*), *A. fumigatus* (*Afu*) and *A. flavus* (*Afla*) (Database: https://fungidb.org, http://smart.emblheidelberg.de/).

The results showed that AFLA_090490 protein, containing one signal peptide at positions 1-18 and two O-glycosyl hydrolase domains (EC 3.2.1) at positions 70-339 and 407-638, was similar to acid trehalase of *S. cerevisiae* and *A. fumigatus* (Figure 1A). AFLA_052430 protein, containing a neutral trehalase calcium-binding domain at position 105-134 and an O-glycosyl hydrolase domain (EC 3.2.1) at position 162-725, was similar to neutral trehalase of *S. cerevisiae* and *A. fumigatus* (Figure 1B). Our findings suggest that *A. flavus* has both acid and neutral trehalases, as seen in *S. cerevisiae* and *A. fumigatus*.

Next, we investigated the ability of *A. flavus* to utilize trehalose as a sole carbon source. The result showed that growth and viability of *A. flavus* on glucose peptone media and trehalose peptone media was similar (Figure 2A and 2B). This finding supports the idea that *A. flavus* utilizes trehalose as a sole carbon source and implies that it degrades extracellular trehalose into glucose for its growth.

Growth inhibition and decreased fungal adherence of *Aspergillus flavus* by validamycin A

To observe the inhibitory effect of validamycin A on *A. flavus* ATCC204304, broth microdilution and XTT assays were performed. The results showed that the minimal inhibition concentration (MIC) of validamycin A against *A. flavus* was 1 μg/mL (Table 1), and the viability of *A. flavus* ATCC204304 after validamycin A treatment at this concentration was significantly decreased when compared to 0.5 μg/mL of validamycin A, 0.25 μg/mL of amphotericin B and the control group (Figure 3).

Next, *A. flavus* ATCC204304 was cultured and treated with or without 0.5 and 1 μg/mL of validamycin A and trehalose levels in the conidia were measured. The results demonstrated that conidia collected from *A. flavus* treated with validamycin A showed significantly higher levels of trehalose than the control (untreated) group, suggesting that validamycin A inhibited trehalase enzymes in the conidia of *A. flavus* (Figure 4A). In addition, the rate of conidial germination was investigated in *A. flavus* conidia treated with 1 μg/mL of validamycin A. The results showed that validamycin A significantly delayed conidial germination of *A. flavus* ATCC204304 particularly at 10 and 12 h (Figure 4B). These data suggest that validamycin A delays conidial germination of *A. flavus* via inhibition of trehalase enzymes.

To observe the effect of validamycin A to exopolysaccharides of *A. flavus*, the crystal violet adherence assays were performed. We observed that 1 μg/mL of validamycin A decreased the adherence property of *A. flavus* ATCC204304 (Figure 4C). These data suggest that validamycin A affects the fungal adherence of *A. flavus*.

Synergistic effects of validamycin A and amphotericin B on *Aspergillus flavus* clinical isolates

232 Antifungal susceptibility tests of *A. flavus* ATCC204304 were performed according to the

233 CLSI broth microdilution method (CLSI M38, 2017). The results demonstrated that the MIC

234 of validamycin A and amphotericin B alone against *A. flavus* ATCC204304 was 1 and 4 µg/mL,

235 respectively (Table 1). Furthermore, the fractional inhibitory concentration index (FICI) was

236 0.625 with the concentrations of validamycin A and amphotericin B at 0.125 µg/mL and 2

237 µg/mL, respectively (Table 1). This finding suggests that validamycin A and amphotericin B

238 have an additive effect on *A. flavus* ATCC204304.

239 To confirm the combinative effects of validamycin A and amphotericin B, *A. flavus*

240 clinical isolates (n=3) with high MICs of amphotericin B (> 4 µg/mL) (Table 1) were chosen

241 to perform checkerboard assays. Interestingly, the FICI was 0.25-0.28, suggesting a synergistic

242 effect between these two drugs on these clinical isolates (Table 1).

243 **No cytotoxicity of validamycin A to human bronchial epithelial cells**

244 Human bronchial epithelial cells, BEAS-2B, were treated with or without validamycin A

245 including amphotericin B at different concentrations. The results demonstrated that 0.125, 0.5,

246 1 µg/mL of validamycin A, 1, 2 µg/mL of amphotericin B, and a combination of these two

247 drugs concentrations of 0.125 µg/mL of validamycin A, and 2 µg/mL of amphotericin B

248 showed no significant cytotoxicity to human bronchial epithelial cells (Figure 5).

249

250 **Discussion**

251 The trehalose pathway is a major mechanism for growth and metabolism of many fungi;

252 however, the presence of trehalase enzymes in many of these fungi is still unknown [19, 21-23,

253 30-32]. Validamycin A is a trehalase enzyme inhibitor produced by *Streptomyces*

254 *hygroscopicus* and is used for fungal inhibition in plants and insects [33, 36, 37, 51, 52]. From

255 many previous reports, in plants and insects, the effect of validamycin A is to inhibit trehalase

256   activity in their cells [53-56]. In a rice fungal pathogen, *Rhizoctonia solani*, validamycin A was

257   shown to inhibit trehalase activity, but not cellulase, pectinase, chitinase, amylase, or

258   glucosidases [57]. Additionally, validamycin A also inhibited the growth of *Rhizoctonia solani,*

259   and *Fusarium culmorum* [33, 38]. However, there are only few studies demonstrating the

260   effects of validamycin A on human fungal pathogens [58]. From our study, we observed that a

261   human fungal pathogen, *A. flavus,* had two trehalase enzymes that shared similar conserved

262   domains and possessed high similarity and identity to *Saccharomyces cerevisiae* and

263   *Aspergillus fumigatus* (Figure 1A&B)*,* including *Rhizoctonia solani* and *Candida albicans*

264   (Figure S1A&B). Therefore, we hypothesize that validamycin A may inhibit trehalase enzyme

265   activity in *A. flavus* similar to previous reports [33, 38, 57].

266   In this study, we investigated the presence of trehalase enzymes, and the effect of the

267   trehalase inhibitor, validamycin A, on the growth of a common pathogenic fungus in humans*,*

268   *A. flavus*. The results showed that *A. flavus* possesses trehalase homologs and grew on trehalose

269   peptone media, similar to growth on glucose peptone media (Figure 2A, B). These findings

270   imply that *A. flavus* utilize trehalase enzymes to degrade trehalose for use as a carbon source

271   and energy. In addition, we observed inhibitory effects of validamycin A on the growth of *A.*

272   *flavus* (Figure 3). This finding suggests that trehalase activity is required for *A. flavus* growth*.*

273   However, direct evidence, such as genetic approaches (e.g. generating trehalase gene-deletion

274   mutants) to support the importance of trehalase is needed to confirm this observation.

275   In a previous study, it was found that validamycin A increased trehalose levels in a

276   pathogenic fungus, *C. albicans* [58]. This result is similar to our findings that showed an

277   increase in trehalose levels of *A. flavus* conidia after validamycin A treatment (Figure 4A).

278   However, further trehalase activity assay using high-performance liquid chromatography

279   (HPLC) is also necessary to confirm the effect of validamycin A against trehalase enzymes in

280   *A. flavus*. As the trehalose pathway is crucial in the early stages of conidial germination [18,

281 19, 47, 59], we further investigated the effect of validamycin A on conidial germination of *A.*

282 *flavus*. Expectedly, validamycin A significantly delayed conidial germination of *A. flavus*

283 (Figure 4B). Therefore, these observations suggest that the inhibition of trehalase enzymes

284 depletes the source of energy and the growth for *A. flavus*. Nonetheless, we observed that

285 conidial germination in the presence of validamycin A was not different from the untreated

286 group at 24-hour incubation. This result suggests that *A. flavus* could probably increase conidial

287 germination by alternative pathways following trehalase inhibition (e.g. mannitol pathway)

288 [60, 61]. A wide variety of different media is still necessary to be further investigated for the

289 trehalose phenotypes in *A. flavus.*

290   In addition, this study further investigated the combinative effect between

291 validamycin A and amphotericin B on *A. flavus* ATCC204304, which is a standard strain for

292 the antifungal susceptibility test. The result demonstrated that these two drugs showed an

293 additive effect on growth inhibition of *A. flavus.* Interestingly, the combination of these drugs

294 had a synergistic effect on *A. flavus* clinical isolates with high MICs of amphotericin B.

295 Although the cutoff value of MIC for amphotericin B resistance in *A. flavus* was unknown,

296 Barchiesi *et al*. suggested that MIC of amphotericin B ≥ 2 µg/mL should be considered as a

297 resistant strain [48, 62].

298   Trehalose pathway is clearly associated with cell wall components, including chitin

299 and beta-glucan, as shown in many previous reports [18, 19, 42, 47]. Disturbance in

300 substrates of trehalose or enzymes or proteins associated with the trehalose pathway in

301 *Aspergillus fumigatus* would lead to changes on the cell wall components and structure [18,

302 19, 42, 47]. Furthermore, trehalose level and proteins associated with the trehalose pathway

303 may affect exopolysaccharide galactosaminogalactans (GAGs), which are important for

304 fungal adherence and biofilm formation, as shown in *A. fumigatus* previous reports [42, 47].

305   In this study, we also observed that validamycin A decreased fungal adherence (Figure 4C).

306   These data imply that the structure or components of exopolysaccharide GAGs may be

307   affected by validamycin A.

308   Besides, trehalase enzymes in many eukaryotic organisms may play important roles in

309   the carbon metabolism, chitin biosynthesis, and stress tolerance, i.e. sucrose and trehalose

310   homeostasis in *Arabidopsis thaliana* and *Phaseolus vulgaris*, regulation of chitin biosynthesis

311   in insects, and carbon partitioning in many plants [63-70]. Therefore, we hypothesize that

312   inhibition of trehalase enzyme via validamycin A may change the structure and components

313   of fungal cell wall and exopolysaccharide through changes in the carbon metabolism of *A.*

314   *flavus* leading to increased permeability and synergistic effects of amphotericin B against *A.*

315   *flavus* in the presence of validamycin A. However, further studies of cell wall/GAGs

316   structures via electron microscope and cell wall/GAGs components through HPLC, including

317   RNA sequencing and metabolomic analyses, are necessary to decipher the effect of

318   validamycin A in *A. flavus* [18, 47].

319   Additionally, MICs of validamycin A in each *A. flavus* clinical isolate were varied.

320   This variation of MICS of validamycin A in these clinical isolates is probably due to the

321   difference in the cell wall/GAGs structure and components of each strain (e.g. glucan or

322   chitin), as a previous study showed that amphotericin B-resistant *A. flavus* contained higher

323   (1,3)-β-D-glucan in their cells wall than the sensitive strains [71]. Furthermore, previous

324   studies suggest that some clinical isolates of *A. fumigatus* had different phenotypes including

325   cell wall components and virulence [72, 73].

326   We further characterized these clinical isolates and observed that the growth rate and

327   conidial trehalose levels showed no difference from *A. flavus* ATCC204304 (Figure

328   S2A&B). However, these isolates possessed different fungal adherence property (Figure

S2C). Different exopolysaccharide components and/or structure of these isolates may lead to decreased permeability of amphotericin B and validamycin A into fungal cell membrane and cytoplasm affecting MICs in each clinical isolate. Nonetheless, the cell wall/GAGs structure and components of these clinical isolates need to be further studied. Moreover, more clinical isolates and animal models are also necessary to confirm synergistic effects between validamycin A and amphotericin B.

Cytotoxicity of validamycin A was tested in our study, and the result demonstrated that validamycin A at concentrations showing synergistic effects on *A. flavus* had no cytotoxicity on human bronchial epithelial cells (Figure 5). Nevertheless, different human cell lines together with different concentrations of validamycin A and amphotericin B are still needed to be further investigated for the cytotoxicity. In addition, *in vivo* studies are required as acute toxicity was found in rodents at very high doses of validamycin A (https://pubchem.ncbi.nlm.nih.gov/compound/Validamycin-A). For future *in vivo* survival studies, different concentrations of validamycin A, i.e. 0.125 and 1 µg/mL with or without the combination of amphotericin B, and different routes of administration, e.g. oral gavage, intraperitoneal route, or intravenous route, are necessary to be further investigated.

In conclusion, this study demonstrated that validamycin A delayed conidial germination and inhibited the growth of *A. flavus*. Moreover, a combination between validamycin A and amphotericin B, showed a synergistic effect on amphotericin B-resistant *A. flavus* clinical isolates. The cytotoxicity of validamycin A to human bronchial epithelial cells was not observed in our study. Therefore, we propose that validamycin A could potentially be used as adjunctive therapy in patients with *A. flavus* infection, particularly those who are infected with amphotericin B-resistant strains.

Data Availability

All data used to support the findings of this study are included within the article and the raw data for each figure are available from the corresponding author upon request.

Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

Supplementary materials

Figure S1. *Aspergillus flavus* **shares similar trehalase enzymes with** *Rhizoctonia solani* **and** *Candida albicans*. A) Percentages of identity and similarity of *AFLA_090490* (B8NLC2) : *R. solani* AGM46811.1 (R4VJL2) and *AFLA_090490* (B8NLC2) : *C. albicans* SC5314 acid trehalase (Q5AAU5) from BLASTp analyses, are 31% identity, 47% similarity, and 32% identity, 48% similarity, respectively. *AFLA*: *Aspergillus flavus*; Glycosyl hydrolase family 65 (Glyco_hydro_65N; Glyco_hydro_65m); Trehalase: Trehalose hydrolysis domain. (Adapted from SMART analyses (http://smart.embl-heidelberg.de/)). B) Percentages of identity and similarity of *AFLA_052438* (B8NS12) : *R. solani* AGM46812.1 (R4VM92) and *AFLA_052438* (B8NS12) : *C. albicans* P78042 neutral trehalase from BLASTp analyses, are 55% identity, 70% similarity, and 55% identity, 71% similarity, respectively. *AFLA*: *Aspergillus flavus*; Trehalase_Ca-bi: Neutral trehalase calcium-binding domain; Trehalase: Trehalose hydrolysis domain. (Adapted from SMART analyses (http://smart.embl-heidelberg.de/)).

Figure S2. Different *Aspergillus flavus* **isolates show no difference in the radial growth rate and conidial trehalose levels, but possess different fungal adherence property.** A) *Aspergillus flavus* ATCC 204304 and three clinical isolates were incubated at 37ºC on glucose media. The radial growth of these fungal growths was measured on the third day of incubation. Data are presented as means ± SE from three biological replicates. No significant difference was observed (one-way ANOVA with post-hoc Bonferroni's test). B) *Aspergillus flavus* ATCC 204304 and three clinical isolates were cultured at 37ºC on Sabouraud dextrose agar for five days with or without 1 μg/mL validamycin A. Trehalose assays were performed to measure trehalose levels in the conidia using glucose oxidase assays. Data are presented as means ± SE from three biological replicates. No significant difference was observed (one-way ANOVA with post-hoc Bonferroni's test). C) *Aspergillus flavus* ATCC 204304 and three clinical isolates were cultured at 37ºC in Sabouraud dextrose broth with or without 1 μg/mL validamycin A in 96-well plates for 24 hours and the crystal violet adherence assays were performed. Data are presented as means ± SE from three biological replicates. *, P-value < 0.05; **, P-value < 0.01 (one-way ANOVA with post-hoc Bonferroni's test compared to ATCC204304 strain).

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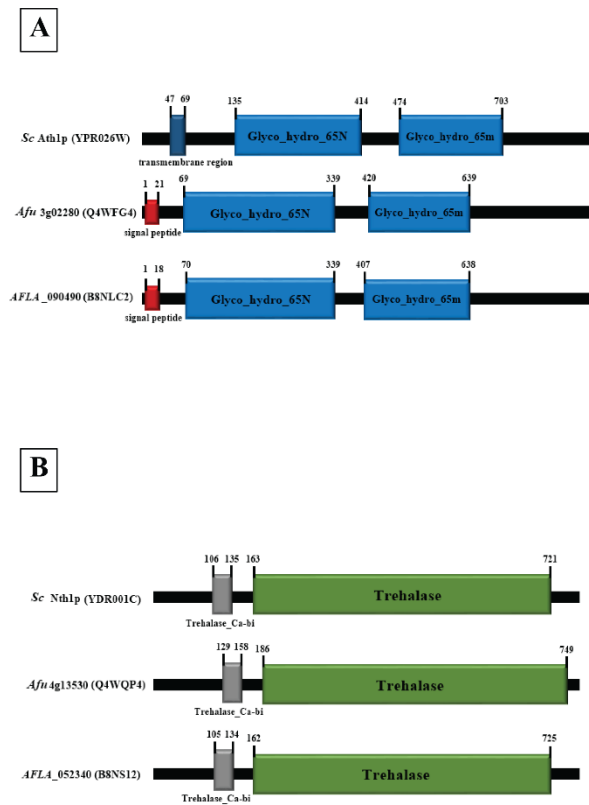
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Figures and table

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Figure 1. *Aspergillus flavus* **possesses trehalase homologs.** A) Percentages of identity and similarity of *Sc*Ath1p (YPR026W) : *AFLA_090490* (B8NLC2) and *Afu3g02280* (Q4WFG4) : AFLA_090490 (B8NLC2) from BLASTp analyses, are 29% identity, 46% similarity, and 68% identity, 81% similarity, respectively. *Sc*Ath1p: *Saccharomyces cerevisiae* acid trehalase protein; *Afu*: *Aspergillus fumigatus*; *AFLA*: *Aspergillus flavus*; Glycosyl hydrolase family 65 (Glyco_hydro_65N; Glyco_hydro_65m). (Adapted from SMART analyses (http://smart.embl-heidelberg.de/)).

B) Percentages of identity and similarity of *Sc*Nth1p (YDR001C) : AFLA_052438 (B8NS12) and Afu4g13530 (Q4WQP4) : AFLA_052438 (B8NS12) from BLASTp analyses, are 55% identity, 69% similarity, and 81% identity, 88% similarity, respectively. *Sc*Nth1p: *Saccharomyces cerevisiae* neutral trehalase protein; *Afu*: *Aspergillus fumigatus*; *AFLA*:

601   *Aspergillus flavus*; Trehalase_Ca-bi: Neutral trehalase calcium-binding domain; Trehalase:

602   Trehalose hydrolysis domain. (Adapted from SMART analyses (http://smart.embl-

603   heidelberg.de/)).

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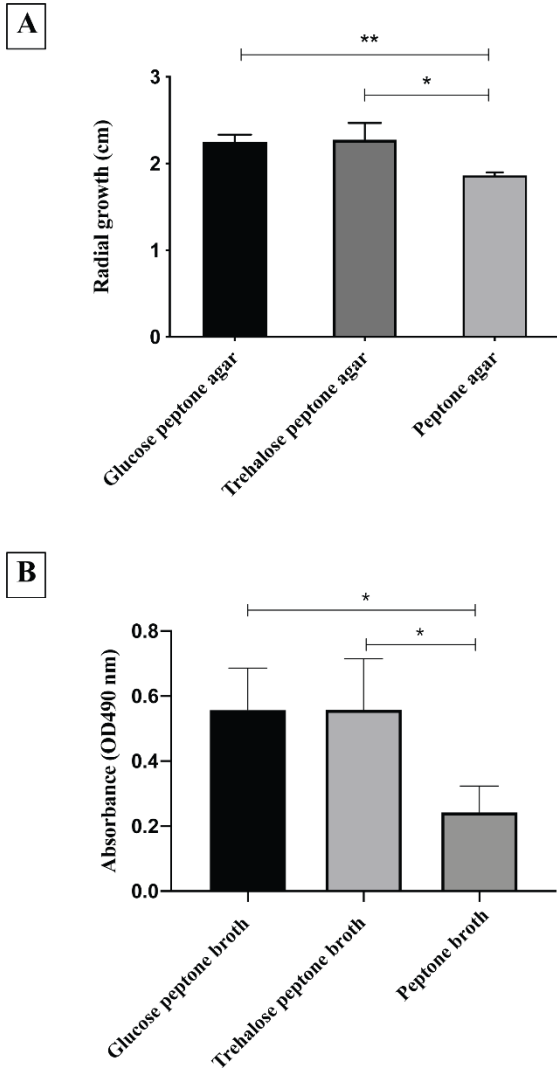
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608   **Figure 2. *Aspergillus flavus* utilizes trehalose as a sole carbon source similar to glucose.**

609   A) *Aspergillus flavus* ATCC 204304 was incubated at 37ºC on glucose peptone, trehalose

610   peptone, and peptone alone media. The radial growth of these fungal growths was measured

611   on the second day of incubation. Data are presented as means ± SE from three biological

612   replicates. *, *P*-value < 0.05; **, *P*-value < 0.01 (one-way ANOVA with post-hoc Bonferroni's

613   test). B) *Aspergillus flavus* ATCC 204304 was incubated at 37°C on glucose peptone, trehalose

614   peptone, and peptone alone liquid media for 24 hours and viability tests using XTT assays were

615   performed. Data are presented as means ± SE from three biological replicates. *, *P*-value <

616   0.05 (one-way ANOVA with post-hoc Bonferroni's test).

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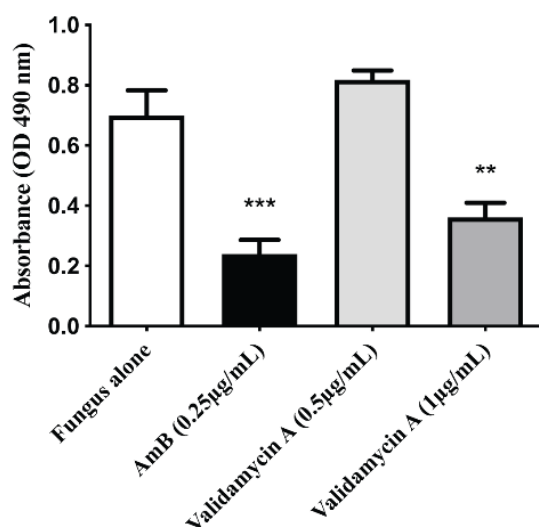
618   **Figure 3. Validamycin A inhibits the growth of *Aspergillus flavus***

619   *Aspergillus flavus* ATCC204304 was cultured at 37°C in RPMI media in 24-well plate for 18

620   hours. Fungal viability was measured by XTT assays at 490 nm. Amp: Amphotericin B at 0.25

621   μg/mL. Data are presented as means ± SE from three biological replicates. *, *P*-value < 0.05;

622   **, *P*-value < 0.01; ***, *P*-value < 0.001 (one-way ANOVA with post-hoc Bonferroni's test

623   compared to fungus alone)

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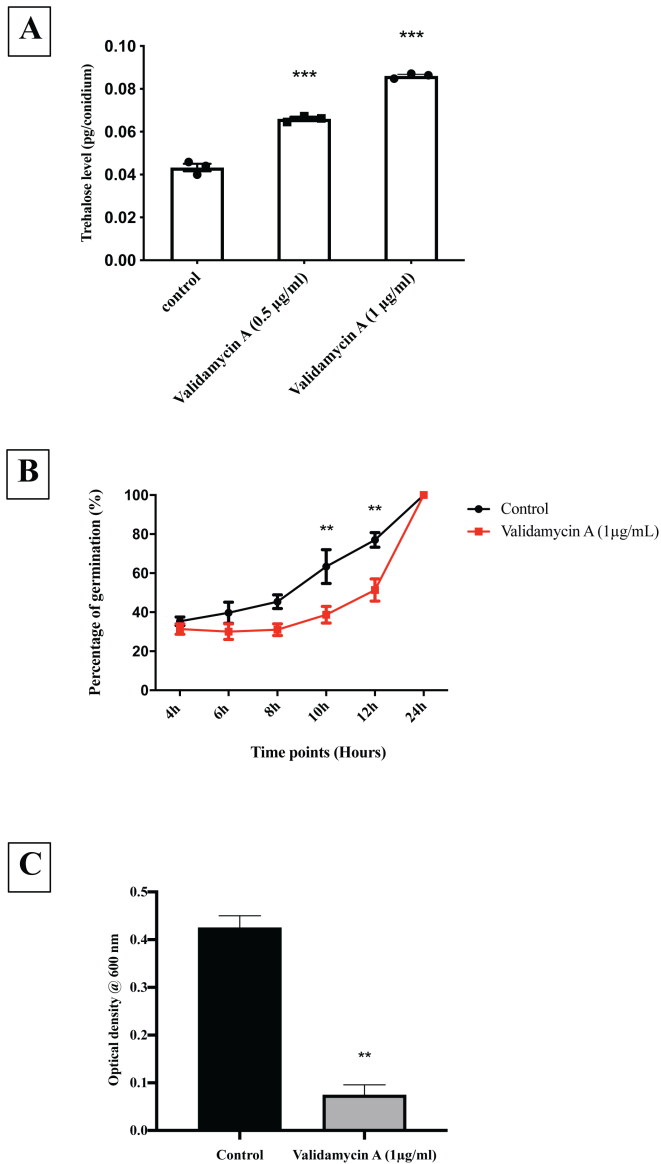
Figure 4. Validamycin A increases trehalose levels in *Aspergillus flavus* conidia with delayed conidial germination and decreased fungal adherence.

A) *Aspergillus flavus* ATCC 204304 was cultured at 37^0C on Sabouraud dextrose agar for five days with or without 1 μg/mL validamycin A. Trehalose assays were performed to measure trehalose levels in the conidia using glucose oxidase assays. Data are presented as means ± SE from three biological replicates. ***, *P*-value < 0.001 (unpaired two-tailed Student's *t*-test compared to the control). B) *Aspergillus flavus* ATCC 204304 was cultured at 37^0C in Sabouraud dextrose broth with or without 1 μg/mL validamycin A in an orbital shaker at 200

633   rpm. Conidial germination at each time point was counted and calculated. Data are presented

634   as means ± SE from three biological replicates. **, *P*-value < 0.01 (unpaired two-tailed

635   Student's *t*-test compared to the control). C) *Aspergillus flavus* ATCC 204304 was cultured at

636   37⁰C in Sabouraud dextrose broth with or without 1 µg/mL validamycin A in 96-well plates

637   for 24 hours and the crystal violet adherence assays were performed. Data are presented as

638   means ± SE from three biological replicates. **, *P*-value < 0.01 (unpaired two-tailed Student's

639   *t*-test compared to the control).

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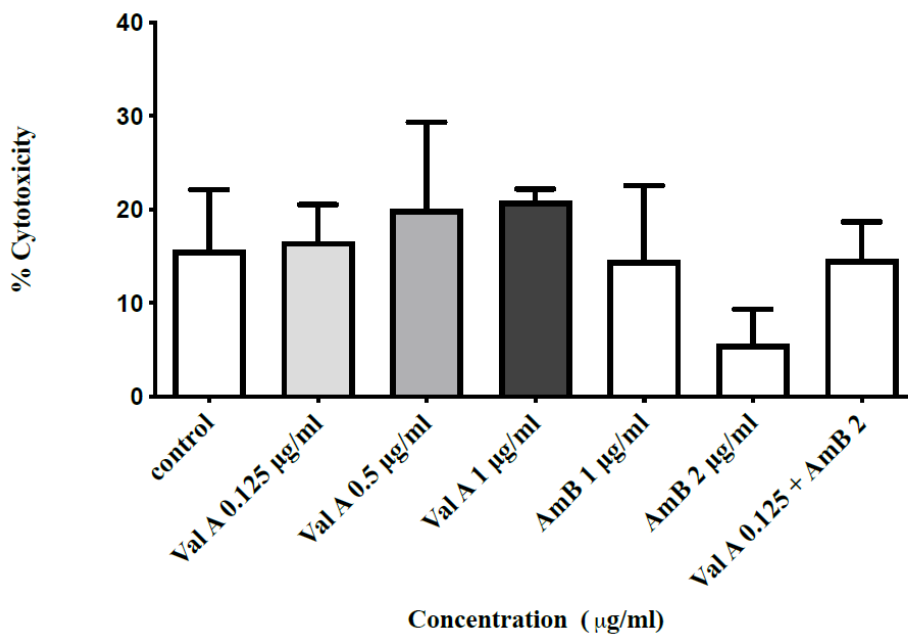
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644   **Figure 5. Validamycin A and the combination of validamycin A and amphotericin B have**

645   **no cytotoxic effect on human bronchial epithelial cells.** The cytotoxicity test was performed

646   to observe the toxicity of validamycin A and amphotericin B on BEAS-2B cells using lactate

647   dehydrogenase (LDH)-Cytotoxicity Colorimetric Assay Kit II. Cell cultures were incubated at

648 37ºC in a humidified environment containing 95% air-5% CO_2. After 24 hours, LDH reaction

649 mixture was added (25 µl), incubated at 37°C for 30 minutes. Then ODs were measured at 450

650 nm using a spectrophotometer. Data are presented as means ± SE from three biological

651 replicates. NS: not significant (one-way ANOVA with post-hoc Bonferroni's test).

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667 **Table 1** Minimum inhibitory concentrations (MICs) of validamycin A alone, amphotericin B alone, or validamycin A in combination with

668 amphotericin B on *Aspergillus flavus* ATCC204304 and *Aspergillus flavus* from clinical isolates. Table also contains patient characteristics, i.e.

669 specimen source, diagnosis, and underlying disease, including the fractional inhibitory concentration index (FICI) and the interpretation of FICI

670 (Interpretation: A: additive; S: synergistic)

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A. flavus strains	Specimen	Diagnosis (EORTC criteria)	Underlying disease	MICs of single agent (µg/mL)		MICs of combined agents (µg/mL)		FICI (µg/mL)	Interpretation
				Validamycin A	Amphotericin B	Validamycin A	Amphotericin B		
A. flavus ATCC204304	Human sputum			1	4	0.125	2	0.625	A
A. flavus SI 1	Left sphenoid sinus	Invasive aspergillosis (Probable invasive aspergillosis)	Diabetes, hypertension, dyslipidemia	>128	8	0.125	2	<0.251	S
A. flavus SP 2	Sputum	Invasive pulmonary aspergillosis (Possible invasive aspergillosis)	Hepatitis C virus cirrhosis	1	8	0.0312	2	0.281	S
A. flavus EN 3	Endotracheal aspiration	Invasive pulmonary aspergillosis (Probable invasive aspergillosis)	Acute lymphoblastic leukemia	>128	8	0.0039	2	<0.250	S