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Gliotoxin effects on fungal growth: Mechanisms and exploitation

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ABSTRACT

Although initially investigated for its antifungal properties, little is actually known about the effect of gliotoxin on *Aspergillus fumigatus* and other fungi. We have observed that exposure of *A. fumigatus* to exogenous gliotoxin (14 μg/ml), under gliotoxin-limited growth conditions, results in significant alteration of the expression of 27 proteins (up- and down-regulated >1.9-fold; $p < 0.05$) including *de novo* expression of Cu, Zn superoxide dismutase, up-regulated allergen Asp f3 expression and down-regulated catalase and a peroxiredoxin levels. Significantly elevated glutathione GSH levels ($p < 0.05$), along with concomitant resistance to diamide, were evident in *A. fumigatus* Δ*gliT*, lacking gliotoxin oxidoreductase, a gliotoxin self-protection gene. *Saccharomyces cerevisiae* deletents (Δ*sod1* and Δ*yap1*) were hypersensitive to exogenous gliotoxin, while Δ*gsh1* was resistant. Significant gliotoxin-mediated (5 μg/ml) growth inhibition ($p < 0.001$) of *Aspergillus nidulans, Aspergillus terreus, Aspergillus niger, Cochliobolus heterostrophus* and *Neurospora crassa* was also observed. Growth of *Aspergillus flavus, Fusarium graminearum* and *Aspergillus oryzae* was significantly inhibited ($p < 0.001$) at gliotoxin (10 μg/ml), indicating differential gliotoxin sensitivity amongst fungi. Re-introduction of *gliT* into *A. fumigatus* Δ*gliT*, at a different locus (*ctsD*; AFUA_4G07040, an aspartic protease), with selection on gliotoxin, facilitated deletion of *ctsD* without use of additional antibiotic selection markers. Absence of *ctsD* expression was accompanied by restoration of *gliT* expression, and resistance to gliotoxin. Thus, we propose *gliT*/gliotoxin as a useful selection marker system for fungal transformation. Finally, we suggest incorporation of gliotoxin sensitivity assays into all future fungal functional genomic studies.

1. Introduction

Production of gliotoxin, an epipolythiodioxopiperazine, by *Aspergillus fumigatus* is enabled by a 28 kb gene cluster (*gli*) located on chromosome 6 (Gardiner et al., 2004; Cramer et al., 2006). The molecule has been intensively studied because of its cytotoxic, immuno-inhibitory and apoptotic effects on animal cells (Sutton et al., 1994). Gliotoxin has recently been shown to inhibit angiogenesis and it has been proposed that this effect prevents tissue neovascularisation, impedes the proinflammatory response and contributes to tissue damage (Ben-ami et al., 2009). As a result of disulphide bridge presence, gliotoxin is a redox active molecule and can cycle between the oxidised and reduced forms depending on the cellular redox state (Waring et al., 1995). In an elegant set of experiments, Bernardo et al. (2003) demonstrated that the oxidised form of gliotoxin is taken up by mammalian cells, undergoes glutathione (GSH)-mediated reduction to the reduced form, which ultimately re-oxidises upon depletion of intracellular GSH and is effluxed from the cell. Deletion of *gliZ* (Bok et al., 2006) and *gliP* (Cramer et al., 2006; Kupfahl et al., 2006), *gliT* (Schrettl et al., 2010; Scharf et al., 2010) and *gliG* (Davis et al., 2011) respectively, abolishes gliotoxin production by *A. fumigatus*. Indeed, Cramer et al. (2006) demonstrated that exogenous gliotoxin controlled the expression of the *gli* cluster in *A. fumigatus* and thereby regulated its own production.

Chamilos et al. (2008) studied the effect of gliotoxin on *Saccharomyces cerevisiae*, using a library of single-gene mutants (4787 strains), in an attempt to further elucidate mechanisms of gliotoxin cytotoxicity and identify novel drug targets in eukaryotic cells. Overall, 10 mutants exhibited increased resistance to gliotoxin while 3 were statistically more sensitive to exogenous gliotoxin, compared to wild-type. Increased resistance to gliotoxin was observed in strains lacking genes encoding metabolic, vesicular transport, DNA damage repair and unknown function functions, while increased sensitivity was evident when Cys3, a cystathionine gamma-lyase and Mef1 a transcriptional elongation factor of mitochondrial origin were absent.

Rohlfs et al. (2007) demonstrated that toxic secondary metabolites produced by *Aspergillus nidulans*, under control of the global regulator *laeA*, significantly discouraged feeding by the fungivore,

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Folsomia candida and proposed that fungivory may select for secondary metabolite biosynthesis in fungi. This led Kwon-Chung and Sugui (2009) to note that production of secondary metabolites might improve the survival chances of fungi. Moreover, although secondary metabolite production may also be influenced by competition between different fungal species, until recently, it was not known if secondary metabolites produced by *Aspergilli* conferred a growth competitive advantage in the presence of related species (Losada et al., 2009). Although these authors identified a number of secreted metabolites, which may contribute to improved survival, no information with respect to individual metabolite potency, or the effect on non-*Aspergillus spp*. was presented. Losada et al. postulated that resistance genes would be required to allow a toxin producer to grow in the presence of its metabolite weaponry and it has now been shown that *gliT* in the *gli* cluster mediates self-protection against exogenous gliotoxin in *A. fumigatus* (Schrettl et al., 2010; Scharf et al., 2010) whereby a *gliT*-deficient strain was unable to grow in the presence of gliotoxin. In fact, *gliT* is the key resistance gene against gliotoxin in *A. fumigatus* and Schrettl et al. demonstrated that Δ*gliT* complementation was possible using selection on gliotoxin-containing media without additional antibiotics (e.g., hygromycin or phleomycin). However, use of *gliT* re-introduction into *A. fumigatus* Δ*gliT*, and subsequent selection in gliotoxin presence, for the targeted deletion of unrelated genes has not been demonstrated.

Thus, a new paradigm has emerged where the effects, and role, of gliotoxin in *A. fumigatus*, in particular, and fungi generally, requires investigation: as it may represent a novel probe to further our understanding of the function of ETPs, and redox control mechanisms, in fungi. In addition, exploitation of the effects of gliotoxin to develop a new fungal selection marker system may be feasible.

2. Materials and methods

2.1. Extraction of gliotoxin from culture filtrates

A. fumigatus ATCC26933 (1×10^5 cfu/ml) was grown for 24 h in either Minimal Essential Medium (plus 5% (v/v) fetal calf serum (MEM/FCS)) or Sabouraud media (25 ml cultures), at 37 °C with shaking at 200 rpm. Supernatants were separated by filtration and an equal volume of extraction buffer (ethyl acetate:chloroform:methanol, 3:2:1) was added. After overnight incubation at 4 °C, filtrates were centrifuged and organic layers removed and evaporated to dryness. Dried extracts were reconstituted in 200 μl HPLC grade Methanol and stored at −70 °C until assay.

2.2. RP-HPLC analysis

Gliotoxin was detected using a reversed phase HPLC (Spectra-Physics). Gradient elution was performed with Solvent A consisting of 0.1% (v/v) trifluoroacetic acid in 5:95 acetonitrile:HPLC grade water (Sigma–Aldrich) and Solvent B consisting of 0.1% (v/v) trifluoroacetic acid in 99.9% acetonitrile. Gliotoxin extracts (20 μl) were injected onto the C_{18} column (Hewlett Packard). All cultures were grown up in triplicate and each one analysed in duplicate. Average values were compared to a standard curve (0–1.0 μg gliotoxin) and gliotoxin quantified as micrograms of gliotoxin per milligram of mycelia produced ±standard deviation.

2.3. Protein extraction

A. fumigatus ATCC26933 (1×10^5 cfu/ml) was grown for 24 h in Sabouraud media (50 ml cultures), at 37 °C with shaking at 200 rpm. After 24 h, gliotoxin (700 μg) dissolved in methanol was added to the cultures ($n = 3$). As a control, methanol only was added to parallel cultures ($n = 3$). After 4 h incubation, mycelia were harvested, filtered under pressure, washed with PBS and resuspended in lysis buffer (100 mM Tris–HCl, 50 mM NaCl, 20 mM EDTA, 10% (v/v) glycerol, 30 mM DTT, 1 mM PMSF and 1 μg/ml pepstatin A pH 7.5; 3 ml of lysis buffer per gram of mycelia). Lysis was accomplished by grinding in liquid N_2 followed by brief sonication on ice. Mycelial lysates were centrifuged (10,000*g*; 30 min) to remove cell debris and the subsequent supernatants analysed by 2D-PAGE following TCA/acetone precipitation (Carberry et al., 2006).

2.4. Protein quantification

Protein was quantified using Bradford reagent (BioRad Laboratories) with BSA used as a standard protein.

2.5. Two-dimensional electrophoresis

Extracts containing 250 μg protein were resuspended in 8 M Urea, 2 M Thiourea, 4% (w/v) CHAPS, 1% (v/v) Triton-X-100, 10 mM Tris-HC1, 65 mM DTT, 0.8% pH 4–7 carrier ampholytes and loaded onto Immobiline Dry strips (GE Healthcare) in the pH range 4–7. Following IEF on an IPGphor II, gels were equilibrated in reducing buffer (50 mM Tris–HCl, 6 M Urea, 2% (w/v) SDS, 30% (v/v) Glycerol, 2% (w/v) DTT, pH 6.8) for 20 min followed by equilibration in alkylation buffer (50 mM Tris–HCl, 6 M urea, 2% (w/v) SDS, 30% (v/v) glycerol, 2.5% (w/v) iodoacetamide, pH 6.8) for a further 20 min. The equilibrated strips were placed on homogenous 12% SDS–PAGE gels and electrophoresed overnight at 100 V using a Protean Xi-II Cell (Bio-Rad Laboratories). Resulting gels were stained with Coomassie Brilliant Blue R and scanned using a Typhoon Trio Variable Mode Imager (GE Healthcare, Freiburg, Germany). Three replicate gels of each, treated and untreated were submitted for analysis using the Image Master Platinum software (GE Healthcare, Freiburg, Germany) to identify differentially regulated spots. The protein spots of interest on each gel were detected, normalised, edited and manually matched to a reference gel (Fig. 1A).

2.6. MALDI-ToF mass spectrometry

Selected proteins were excised from 2D-PAGE gels using an automated spot cutter (GE Healthcare, Freiburg, Germany), digested with trypsin and analysed as previously described (Carberry et al., 2006). Mass spectrometry was carried out using an Ettan™ MALDI-ToF mass spectrometer (GE Healthcare, Freiburg, Germany), internal calibrants, Angiotensin III (Sigma–Aldrich) and ACTH fragment 18–39 (Sigma–Aldrich), were used to calibrate all spectra. Protein identification was carried out either by m/z data interrogation of a (I) local FASTA version of the annotated *A. fumigatus* genome available at http://www.cadre-genomes.org.uk/ and (II) Mascot™ nrNCBI database (Mabey et al., 2004).

2.7. GSH/GSSG and superoxide dismutase activity determination

Intracellular glutathione was measured using the method of Thön et al. (2010) and Rahman et al. (2006), with modifications. *A. fumigatus* ATCC26933 and Δ*gliT* (Schrettl et al., 2010), respectively, were cultured for 21 h in AMM before addition of gliotoxin (5 μg/ml final) for 3 h. Mycelia were harvested through miracloth and dried. Mycelia (500 mg) in 5% (w/v) SSA (500 μl) were bead-beaten at 30 Hz for 5 min followed by centrifugation at 12,000*g* for 10 min at 4 °C. Supernatants were removed, covered in tinfoil and neutralised using triethanolamine. All samples were diluted (1/10–1/30) in 125 mM sodium phosphate, 6.3 mM EDTA, pH 7.5 prior to centrifugation at 12,000 g for 10 min at 4 °C. Whole cell