Review

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Resistance is not futile: gliotoxin biosynthesis, functionality and utility

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Gliotoxin biosynthesis is encoded by the *gli* gene cluster in *Aspergillus fumigatus*. The biosynthesis of gliotoxin is influenced by a suite of transcriptionally-active regulatory proteins and a *bis*-thiomethyltransferase. A self-protection system against gliotoxin is present in *A. fumigatus*. Several additional metabolites are also produced via the gliotoxin biosynthetic pathway. Moreover, the biosynthesis of unrelated natural products appears to be influenced either by gliotoxin or by the activity of specific reactions within the biosynthetic pathway. The activity of gliotoxin against animal cells and fungi, often mediated by interference with redox homeostasis or protein modification, is revealing new metabolic interactions within eukaryotic systems. Nature has provided a most useful natural product with which to reveal some of its many molecular secrets.

Contextualizing and rethinking gliotoxin

Aspergillus fumigatus is an opportunistic fungal pathogen and primarily infects immunocompromised individuals where it can cause fatal invasive aspergillosis (IA) [1]. *A. fumigatus* exposure can also induce debilitating aspergillosis and allergy in immunocompetent individuals [2,3]. Selected secondary metabolites produced by *A. fumigatus*, in particular siderophores and the non-ribosomal peptide gliotoxin, are generally considered to be front-line virulence factors [4,5]. Gliotoxin is an epipolythiodioxopiperazine (ETP) of molecular mass 326 Da, and contains a disulfide bridge which can undergo repeating cleavage and reformation, thereby resulting in a potent intracellular redox activity (Figure 1) [6]. Indeed, the dithiol form of gliotoxin has also been posited to be responsible for the observed biological activities of gliotoxin [7]. *Bis*dethio*bis*(methylthio)gliotoxin (BmGT) and related gliotoxin metabolites (Figure 1) are also biosynthesized by *A. fumigatus* [8,9]. Incredibly, the gliotoxin biosynthetic pathway had remained elusive since the discovery of gliotoxin in 1936; however, recent studies have not only dissected this unusual molecular assembly system but have revealed the necessity for gliotoxin-producing fungi to possess an endogenous resistance system against gliotoxin [10,11]. Moreover, because gliotoxin can be considered as the prototype ETP, studies on gliotoxin can be instrumental in revealing the biosynthetic mechanisms of related ETPs which are biosynthesized by a range of fungi [12]. Amongst others, these include sirodesmin A, sporidesmin A, chaetocin, aranotin, and chetomin [13]. Studies on the biosynthetic mechanism of ETPs, particularly gliotoxin, are also serving to inspire new synthetic chemistry approaches for ETP synthesis and desulfurization, which are somewhat beyond the scope of the present review [13,14].

In addition to studying how gliotoxin contributes to organismal virulence, it has also been deployed to explore and reveal novel biochemistry within both fungal and animal cells [15,16]. Thus, we contend that it is the ability of gliotoxin to interfere with so many cellular processes that makes it such a useful tool to access the many important, although occluded, systems interactions within fungi. Indeed, emerging studies of the reciprocal effects of gliotoxin on animal cells and fungi are serving to enhance our view of gliotoxin as an antioxidant as much as a toxin [6,17]. In this review we set out our current understanding of gliotoxin biosynthesis, its regulation, and resistance systems against gliotoxin. We also attempt to elucidate how the effects of gliotoxin on fungi and animal cells can cross-fertilize our knowledge of hitherto unknown aspects of both eukaryotic cell systems.

Gliotoxin biosynthesis

The *in silico* identification of the *gli* cluster in *A. fumigatus* (Figure 1) [18] paved the way for multiple functional genomic studies which revealed that the *gli* cluster, comprising 13 genes, encodes the key enzymes responsible for gliotoxin biosynthesis (Figure 2). The $Zn(II)_2$-Cys(6) binuclear cluster domain transcription factor *gliZ* was confirmed as the regulator of the *gli* cluster [19], whereby deletion of this gene abrogated the expression of key biosynthetic genes and concomitant gliotoxin biosynthesis. Interestingly, upon *gliZ* reconstitution in *A. fumigatus* Δ*gliZ*, the biosynthesis of unrelated natural products was induced. The non-ribosomal peptide synthetase, GliP, catalyzes the formation of cyclo-phenylalanyl-serine, the first biosynthetic intermediate in the gliotoxin biosynthetic pathway [20], and multiple groups undertook *gliP* deletion, which results in complete loss of gliotoxin biosynthesis [5,21–23] (Figure 2). Interestingly, Cramer *et al*. observed that exogenous gliotoxin induced *gli* cluster gene expression in *A. fumigatus*, which was somewhat attenuated in *A. fumigatus* Δ*gliP* [21]. Although not unheard of, it is certainly unusual for a fungal natural product to induce its own biosynthesis, and the biological significance of this

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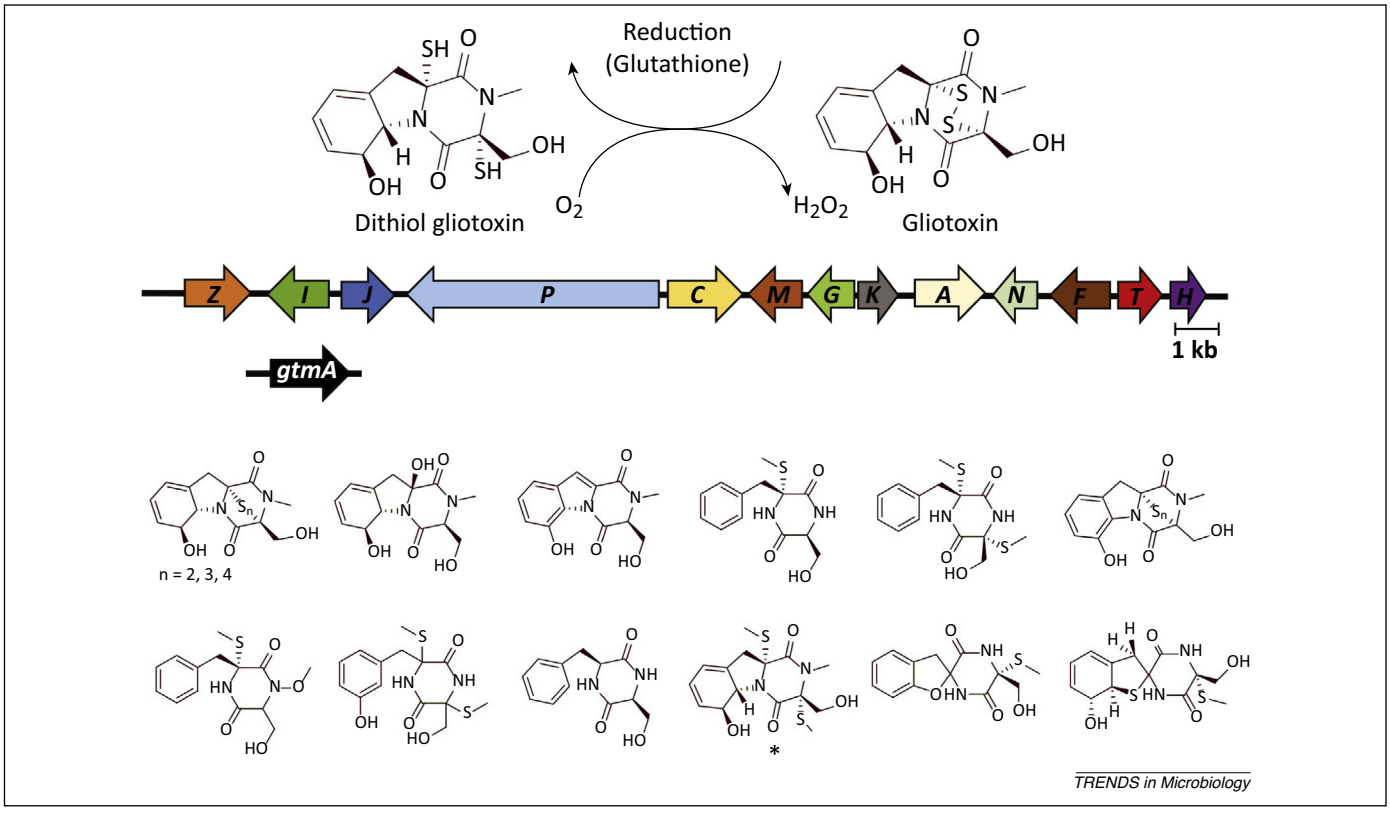

Figure 1. Interconversion of gliotoxin between the reduced (dithiol) and oxidized (disulfide) forms. In *Aspergillus fumigatus* the *gli* cluster, which encodes gliotoxin biosynthesis and consists of 13 genes (in color and labeled with their last letter), is located on chromosome 6 [10,18]. *gtmA* is encoded outside the cluster and is on chromosome 2. Multiple metabolites are produced via the activity of the gliotoxin biosynthetic pathway (shown at the bottom of the figure), including BmGT (indicated by an asterisk) [9].

phenomenon is only beginning to emerge. Cyclo-phenylalanyl-serine is subsequently converted to a highly-reactive acyl imine intermediate before *bis*-glutathionylation. GliC has been shown to catalyze hydroxylation of the α-carbon of L-Phe in cyclo-phenylalanyl-serine before *bis*-glutathionylation [24]. The demonstration that *gliG* encodes a glutathione *S*-transferase (GST), which conjugates two glutathione (GSH) molecules to a biosynthetic intermediate to form a *bis*-glutathionylated biosynthetic intermediate (Figure 2), and which is therefore responsible for the sulfurization of gliotoxin, was an important finding because it was one of the first demonstrations that GSTs played a biosynthetic, as opposed to detoxification, role in fungi [25,26]. This *bis*-glutathionylated intermediate is subsequently processed by GliK, a γ-glutamyl cyclotransferase, to remove both γ-glutamyl moieties [17,27]. Subsequent processing via GliI [28] yields a biosynthetic intermediate, which is *N*-methylated via the *N*-methyltransferase GliN, before the gliotoxin oxidoreductase GliT-mediated disulfide bridge closure (Figure 2) [27,29]. Gliotoxin secretion from *A. fumigatus* ensues, now known to be facilitated in part by the MFS transporter GliA [30].

Regulation of gliotoxin biosynthesis

Dolan *et al*. have identified a negative regulatory mechanism which deploys a non-*gli* cluster encoded gliotoxin *bis*-thiomethyltransferase, termed GtmA [8]. *gtmA* expression is induced by gliotoxin, and GtmA (also termed TtmA in [29]) functions to *bis*-thiomethylate dithiol gliotoxin to form BmGT [8,29], instead of undergoing disulfide bridge closure via GliT (Figure 2). Consequently, both gliotoxin biosynthesis and auto-induction of the *gli* cluster are attenuated. Importantly, this post-biosynthetic negative regulatory mechanism may be operative in other fungal ETP biosynthetic systems because *bis*-thiomethylated ETPs are produced by fungi, and GtmA orthologs have also been identified across the ascomycetes [8,29]. Moreover, overproduction of gliotoxin or related ETPs for biotechnological purposes may be dependent on utilizing *bis*-thiomethyltransferase-deficient strains to deactivate this negative feedback system. Although GliH has been shown to be essential for gliotoxin biosynthesis [10], to date the function of this enzyme has not been elucidated.

Interestingly, apart from *gliZ*, many other transcription factors and regulatory genes influence gliotoxin biosynthesis (Table 1). The C_2H_2 transcription factor *gipA* regulates *gliA* interdependently with *gliZ* [31]. In addition, it is well-established that *laeA*, a global regulator of secondary metabolism, which interacts with the velvet proteins VeA and VelB in the nucleus [32–35], augments gliotoxin biosynthesis because either *laeA* or *veA* deletion results in impaired gliotoxin production in *A. fumigatus* [34,36]. Other transcription factors found to regulate the *gli* cluster include *mtfA* (a C_2H_2 transcription factor [37]), *rsmA* (a bZIP transcription factor [38]), and *stuA* (an APSES family transcription factor [39,40]) (Table 1). The developmental regulators *flbB* and *flbE* positively influence gliotoxin biosynthesis because deletion mutants exhibit reduced or abolished gliotoxin production [41–43]. The

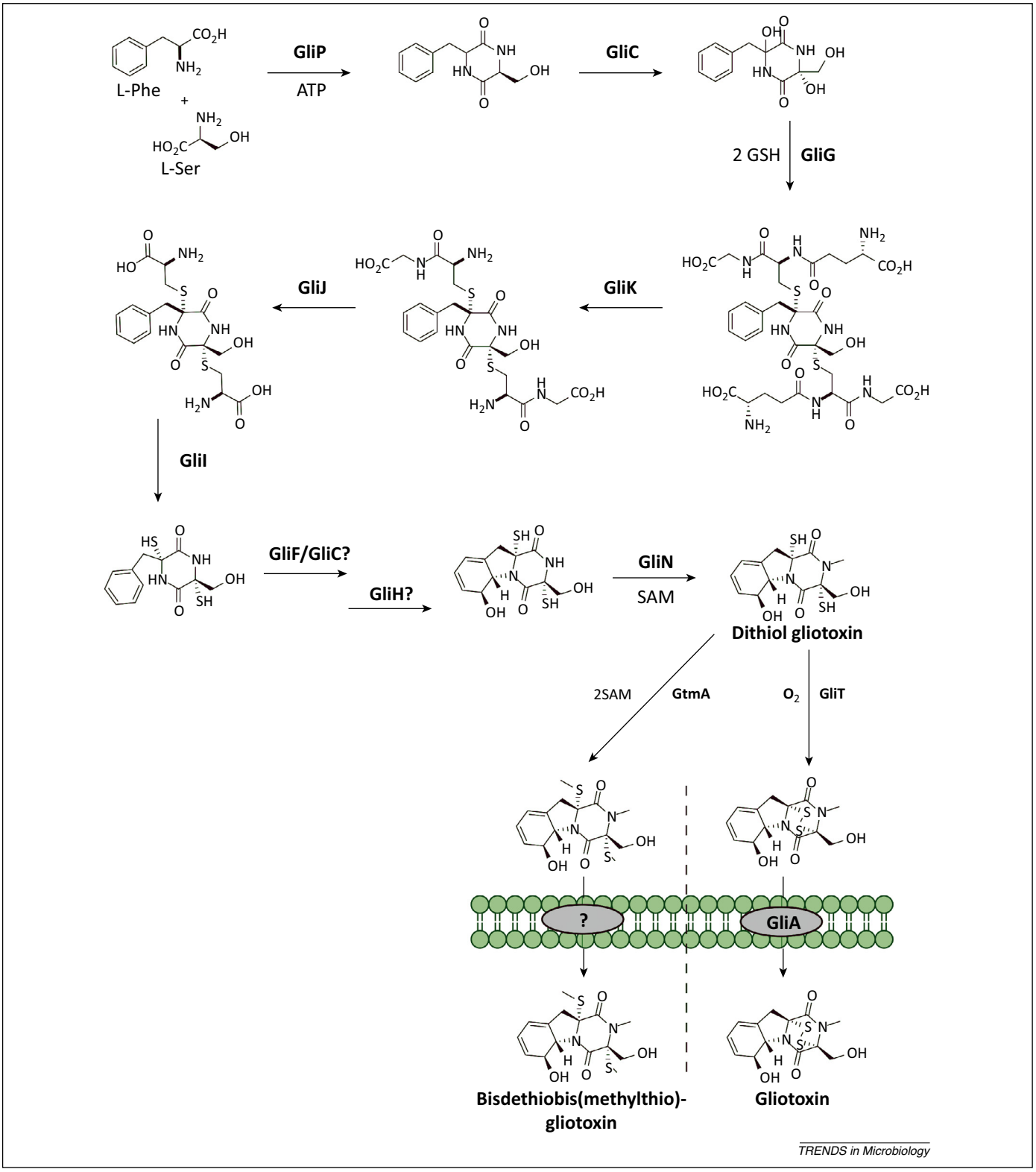

Figure 2. Gliotoxin biosynthesis, secretion, and regulation. A series of enzyme-catalyzed steps convert phenylalanine and serine to gliotoxin or BmGT via the formation of reactive intermediates, *bis*-glutathionylation, degradation steps, methylation, and oxidation [10,11,15,20,25,26]. Dithiol gliotoxin, a co-substrate for either GliT or GtmA, is converted to gliotoxin or BmGT, respectively [8,83]. Gliotoxin can induce *gli* cluster expression [21] or is secreted from *A. fumigatus* via GliA [30]; however, the process of BmGT release is undefined. BmGT formation and secretion dissipate intracellular gliotoxin, thereby preventing *gli* cluster expression leading to downregulation of gliotoxin biosynthesis [8]. Please note that the precise order of some reaction steps, and *gliM*/*H* involvement, remain to be fully elucidated.

MAP kinase, *mpkA*, and the class 2 histone deacetylase, *hdaA*, also positively regulate gliotoxin biosynthesis while the histone methyltransferase, *cclA*, negatively regulates gliotoxin production because increased gliotoxin levels were evident in Δ*cclA*, consequent to increased *gliZ* expression [44] (Table 1). Thus, it is now apparent that regulation of gliotoxin biosynthesis is a hierarchical, multifactorial process that is affected by many regulatory elements, and which is highly integrated into *A. fumigatus* metabolism.

Table 1. Transcriptional regulators which impact upon *gli* cluster gene expression and gliotoxin biosynthesis

Transcriptional regulator	Effect on gliotoxin biosynthesis[a]	Other phenotypes	Refs
GliZ (Zn_2Cys_6 binuclear transcription factor)	Essential for gliotoxin biosynthesis. Deletion results in loss of expression of some of the other *gli* cluster genes (e.g., *gliI*, *gliA*, *gliG*).	*gliZ* regulates the production of other SM (e.g., OE*gliZ* produced helvolic acid at 37°C, unlike wild type). Virulence in a mouse model is not significantly different from wild type, although is slightly decreased. Involved in apoptotic but not necrotic cell death.	[10,19]
GipA (C_2H_2 transcription factor)	Regulates gliotoxin biosynthesis genes; decreased expression of *gliA*, *gliZ*, *gliP*, and *gliT*, and consequently reduced gliotoxin production in Δ*gipA*. Conversely, these genes had increased expression in OE*gipA*. The *gli* genes (except *gliM*) contain potential GipA binding sites. GipA cannot induce *gliA* or *gliP* expression independently of *gliZ*, while *gliZ* induction of *gliA* is GipA-dependent.	GipA potentially induces other SM clusters because at least one gene from 18 SM clusters was increased in expression in an OE*gipA* strain.	[31]
LaeA (transcriptional regulator)	Positively regulates gliotoxin biosynthesis because gliotoxin production is impaired in Δ*laeA*.	Global regulator of SM production in *A. fumigatus* because 13 of 22 SM clusters exhibited reduced expression in Δ*laeA*. Δ*laeA* had reduced virulence in a mouse model of IA. Δ*laeA* conidia were defective in rodlet production and had increased susceptibility to macrophage phagocytosis, while hyphae displayed decreased ability to kill neutrophils. Δ*laeA* demonstrated impaired conidia and conidiophore production in liquid shake culture.	[34,35]
VeA (regulatory gene)	Positively regulates gliotoxin biosynthesis because reduced gliotoxin production is evident in Δ*veA*. *gliZ* and *gliP* expression are decreased in Δ*veA*.	VeA predominantly positively regulates other SM clusters; fumagillin, fumitremorgin G, fumigaclavine C, and glionitrin A production is reduced in Δ*veA*. VeA regulates development; Δ*veA* demonstrates reduced conidiation but increased pigmentation of conidia, reduced protease activity, and increased expression of *brlA* (a developmental activator) during development compared to wild type. VeA is dispensable for virulence in a neutropenic mouse infection model. VeA is highly conserved; *veA* from *Aspergillus nidulans* complements deletion in *A. fumigatus*.	[36,84,85]
FlbA (regulator of G-protein signaling protein)	Does not affect gliotoxin biosynthesis; however, expression of *gliT* and secretion of GliT are increased in Δ*flbA*. Consequently, Δ*flbA* displays enhanced tolerance to exogenous gliotoxin.	*laeA* expression is reduced in Δ*flbA*, suggesting that other SM clusters may be effected by FlbA. FlbA is necessary for normal cell death progression and autolysis in submerged cultures. Δ*flbA* has increased superoxide dismutase and catalase activity, and demonstrated enhanced resistance to menadione and paraquat. FlbA downregulates hyphal differentiation by inactivating GpaA, and thus induces asexual development.	[86,87]
MpkA (MAP kinase)	Regulates gliotoxin production which is reduced in Δ*mpkA* compared to wild type. Expression of *gliN* and *gliT* is decreased in Δ*mpkA*.	MpkA is activated by iron starvation, to which it responds by reshuffling the amino acid pool (increased ornithine levels) leading to increased siderophore biosynthesis. Δ*mpkA* exhibits increased sensitivity to menadione and diamide but decreased sensitivity to H_2O_2. Involved in cell wall integrity signaling.	[88,89]
MtfA (C_2H_2 transcription factor)	Gliotoxin levels were increased in OE*mtfA* but were unchanged in Δ*mtfA*. Expression of *gliZ* and *gliP* was increased in OE*mtfA*.	Involved in growth and development; reduced growth and conidiation was observed in Δ*mtfA* and OE*mtfA*. Positive regulator of protease production. Δ*mtfA* exhibited attenuated virulence in *Galleria mellonella*.	[37]
RsmA (restorer of secondary metabolism – A bZIP transcription factor)	The production of 12 *gli* cluster metabolites was increased 2–100-fold in the OE*rsmA* strain compared to wild type.	Involved in growth and oxidative stress resistance; OE*rsmA* exhibits a growth defect at low growth temperature (25°C). OE*rsmA* mutants show increased resistance to menadione.	[38]
CclA (histone methyltransferase)	Increased gliotoxin production in Δ*cclA* mediated by a substantial increase in *gliZ* transcription.	Increased production of other unidentified secondary metabolites. Severe growth defects and sensitivity to 6-azauracil (6AU); an inhibitor of guanine nucleotide synthesis and an indicator of transcriptional defects.	[44]

Table 1 (*Continued*)

Transcriptional regulator	Effect on gliotoxin biosynthesis[a]	Other phenotypes	Refs
FlbB [developmental regulator containing a basic leucine-zipper domain (bZIP)]	Drastically reduced gliotoxin production (~85%) in the Δ*flbB* mutant compared to wild type.	Delayed/reduced sporulation; precocious cell death; lack of conidiophore development in submerged culture, reduced (~90%) fumagillin production.	[42,90]
FlbE (developmental regulator interacts with FlbB)	Gliotoxin production was abolished in the Δ*flbE* deletion strain.	Controls asexual development: deletion results in reduced conidiation and delayed expression of *brlA* and *vosA*. Necessary for salt-induced development in liquid submerged culture.	[43,90]
HdaA (class 2 histone deacetylase)	Deletion of the *hdaA* gene decreased gliotoxin production and overexpression of *hdaA* increased gliotoxin production.	Δ*hdaA* demonstrated a reduction in both germination rate and vegetative growth; altered colony morphology and increased expression of several NRPS genes.	[50]
StuA (APSES family transcription factor)	*gliP* expression, and likely gliotoxin production, are dependent on StuA *in vivo*.	Δ*stuA* had severely impaired conidiation; six secondary metabolite clusters were found to be regulated by StuA.	[39,40]

[a]Abbreviations: IA, invasive aspergillosis; OE, overexpressed; SM, secondary metabolite.

Histone H3 lysine 9 (H3K9) methyltransferase inhibition

Gliotoxin, and indeed other ETPs (e.g., chetomin and chaetocin), are inhibitors of H3K9 methyltransferases, which has been demonstrated using recombinant enzymes [45,46]. The disulfide bond of ETP molecules is crucial for this inhibition because ETPs in which the thiols are rendered unreactive (e.g., *bis*-dethiobisacetylgliotoxin and chaetocin with methylated sulfur groups) do not display inhibitory abilities [45,46]. While direct investigations of the effects of gliotoxin on histone H3K9 methyltransferases in *A. fumigatus* or other filamentous fungi have not been performed to the best of our knowledge, functional studies of histone-modifying enzymes have been carried out (reviewed in [47]). Disruption of histone modifications, in particular methylation and acetylation of histone H3 tail residues, has been linked with alterations in not only gliotoxin production but also in secondary metabolite production in general in the *Aspergilli* [47–49]. A member of the conserved eukaryotic H3K4 methylating COMPASS complex, CclA, is required for tri- and di-methylation of H3K4 and regulates secondary metabolite production [44,48]. Deletion of *cclA* resulted in increased production of gliotoxin and other secondary metabolites, leading to the conclusion that tri- and di-methylation of H3K4 play a part in secondary metabolite production in *A. fumigatus* [44]. In addition, the class 2 histone deacetylase, HdaA, also regulates secondary metabolite production – deletion was shown to increase production of several secondary metabolites but decrease the production of gliotoxin, while conversely a strain overexpressing *hdaA* had increased gliotoxin biosynthesis [50]. Interestingly, H3K9 methylation levels were slightly higher in Δ*hdaA* compared to wild type. Undoubtedly, further work will be necessary to explore the interactions, if any, between histone modification and gliotoxin biosynthesis.

Self-protection against gliotoxin

The presence of cytochrome P_{450} monooxygenases and GST involvement in ETP, specifically gliotoxin, biosynthesis is reminiscent of a phase I/II detoxification system (C. Davis, PhD thesis, National University of Ireland Maynooth, 2011) [26]. Consequently, it is plausible that gliotoxin biosynthesis evolved from an ancestral detoxification system and that gliotoxin, or a biosynthetic intermediate, conferred a significant competitive advantage on the host organism. Indeed, it is now clear [25,26] that *A. fumigatus* sacrifices reducing power (2 GSH) to enable gliotoxin biosynthesis, and it is therefore not inconceivable that enhanced redox homeostasis, antimicrobial defense, or even redox-mediated signaling, as a consequence of gliotoxin biosynthesis – and presence – drove cluster evolution. However, the selective advantage conferred upon ETP-producing organisms came at a price, and self-protection against redox-active molecular species is also an essential characteristic in these species. Specifically, in addition to effecting disulfide bridge closure during gliotoxin biosynthesis, GliT has been shown to confer resistance against exogenous gliotoxin [10,11] (Figure 3). Moreover, *gliT* expression is induced by gliotoxin independently of *gliZ* [10], which further underpins its essential role in self-protection. Interestingly, the alternative transcription factor which influences *gliZ*-independent *gliT* expression remains elusive.

GliA-mediated gliotoxin efflux from *A. fumigatus* contributes to self-protection [30] (Figures 2 and 3), and SirA, a GliA ortholog in *Leptosphaeria maculans*, likewise confers resistance to exogenous sirodesmin [51]. *A. fumigatus*

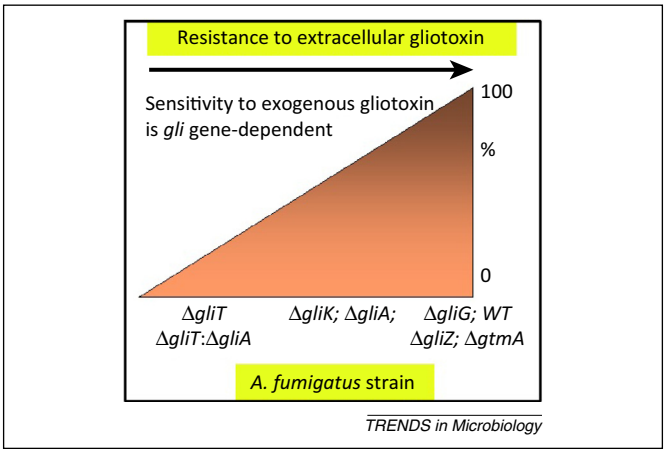

Figure 3. Differential resistance to extracellular gliotoxin following selected *gli* cluster gene and *gtmA* deletion in *Aspergillus fumigatus*. Combined deletion of gliotoxin oxidoreductase *gliT* and MFS transporter *gliA* results in the most gliotoxin-sensitive deletion strain [30], along with Δ*gliT*. *A. fumigatus* wild type (WT), Δ*gliG*, Δ*gtmA* and Δ*gliZ* exhibit intact gliotoxin-resistance, while Δ*gliK* and Δ*gliA* exhibit intermediate sensitivity.

Δ*gliA*:Δ*gliT* is also highly sensitive to exogenous gliotoxin (Figure 3), and this led Wang *et al*. [30] to conclude that they represent independent self-protection systems. However, an alternative hypothesis whereby GliT-mediated gliotoxin oxidation is necessary for GliA-mediated efflux is also compatible with their experimental observations. These authors also observed that attenuated gliotoxin levels are secreted by *A. fumigatus* Δ*gliA* and they posited the existence of an alternative gliotoxin efflux mechanism. However, demonstration of a supplementary secretion system remains outstanding. The identification of *bis*-thiomethylated holomycin in the bacteria *Streptomyces clavuligerus*, and its role in deactivation of the dithiol 'warhead', led to speculation about the presence of a back-up self-protection system catalyzed by *gliM*/*gliN* (methyltransferases) encoded within the *gli* cluster in *A. fumigatus* (Figure 1) [52]. However, it appears that GtmA is the sole enzyme responsible for BmGT formation in *A. fumigatus* and that its primary role is the diminution of gliotoxin biosynthesis and not as a back-up self-protection system involving the direct detoxification of dithiol gliotoxin via *bis*-thiomethylation [8]. Indeed, deletion of *gtmA* has no significant impact on sensitivity of *A. fumigatus* to exogenous gliotoxin (Figure 3), which implies that GliT is the key mediator of this self-protection system.

The gliotoxin-related metabolome

Increasingly, examples of fungal secondary metabolite cross-talk, whereby abrogation or overproduction of one secondary metabolite may affect the production of another apparently unrelated metabolite are emerging, as reviewed in [53]. This phenomenon has been described in *A. fumigatus* in relation to gliotoxin biosynthesis, whereby overexpression of *gliZ* led to helvolic acid production at 37°C which was not observed in wild type, while the production of multiple metabolites was altered by the loss or gain of *gliZ*, suggesting that *gliZ* may influence the biosynthesis of secondary metabolites other than gliotoxin [19]. In a later study employing 2D NMR spectroscopy (DANS) which compared metabolite extracts from wild type and Δ*gliZ*, nine novel *gliZ*-dependent metabolites were identified in addition to previously identified metabolites [9] (Figure 1).

Disruption of gliotoxin biosynthesis in *A. fumigatus* Δ*gliK* prevents the degradation and recycling of 2 mol GSH per mol gliotoxin produced, and this resulted in the concomitant acquisition of an oxidative stress phenotype accompanied by the first identification of the antioxidant ergothioneine (EGT) in *A. fumigatus* [17]. Moreover, significantly elevated EGT levels, possibly due to altered redox homeostasis, were evident in *A. fumigatus* Δ*gliK* [17]. The systems significance of the interactions between these three redox-active species, GSH, gliotoxin, and EGT, remains to be elucidated. In addition, significantly increased levels of two unrelated metabolites of *m*/*z* 394 and 396 were also reported, implying that disruption of gliotoxin biosynthesis, specifically via *gliK* deletion, influences the production of other, unrelated natural products [17]. Further evidence of this phenomenon was observed in *A. fumigatus* Δ*gliT* where production of brevianamide F was significantly increased, while production of tryprostatin A and B, fumagillin, and pseurotin A was significantly decreased [54]. This suggests that although GliT-mediated disulfide bridge closure is required for gliotoxin biosynthesis, abrogation of this redox reaction dysregulates the biosynthesis of other secondary metabolites, raising the question as to whether this is the case in other microbial species with similar disulfide bridge-forming systems (e.g., holomycin in *Streptomyces clavuligerus* and *Yersinia ruckeri*) [55,56]. In any case, it is now clear that disruption of gliotoxin biosynthesis in *A. fumigatus* is a *trans*-consequential event which has far-reaching effects on secondary metabolism, possibly as a result of *A. fumigatus* compensating for the loss of gliotoxin-influenced redox homeostasis.

Gliotoxin functionality in fungi

With the discovery of GliT as the key to *A. fumigatus* self-protection against gliotoxin toxicity [10,11], and the demonstration of how gliotoxin can inhibit growth of *Aspergilli* and pathogenic fungi such as *Candida albicans* [57,92], the intriguing questions of the true *in vivo* role(s) of this molecule and the *in vivo* effects upon fungi, either gliotoxin producers or not, was brought to the fore. While the exact mode of action of gliotoxin *in vivo* remains to be fully elucidated, studies in yeast, *Aspergillus spp*., and mammalian cell lines have demonstrated that elevated intracellular levels of GSH can exacerbate toxicity [15,58,59], effectively by reducing the gliotoxin disulfide bridge to yield reactive dithiol gliotoxin and thus allowing many as-yet unidentified detrimental interactions to occur between reduced gliotoxin and essential cellular components. The importance of elevated GSH levels in mediating gliotoxin toxicity in fungi is particularly highlighted by the ability of yeast deleted for *gsh1* (the gene encoding γ-glutamylcysteine synthetase which is essential for GSH biosynthesis) to grow much better than wild type in the presence of high levels of exogenous gliotoxin, and by the elevated GSH levels in gliotoxin-sensitive *A. fumigatus* Δ*gliT* [15].

In an attempt to gain insight into the *in vivo* effects of gliotoxin exposure, Chamilos and colleagues [60] screened a yeast deletion library to identify strains that were sensitive or resistant to exogenous gliotoxin compared to wild type. While a number of gene deletions caused changes in the gliotoxin exposure profile, of particular note was the finding that deletion of *cys3*, which encodes a cystathionine γ-lyase, caused sensitization to gliotoxin. Cys3 is the second enzyme in the reverse transulfuration pathway and is responsible for converting homocysteine, a product of *S*-adenosylmethionine metabolism, to cysteine [61]. Given recent studies demonstrating the importance of GSH in mediating gliotoxin sensitivity [15,17], and a recent global transcriptome study demonstrating major alterations in sulfur metabolism genes in *A. fumigatus* Δ*gliT* upon gliotoxin exposure [54], the finding that *cys3* deletion sensitizes yeast to gliotoxin alludes to evolutionarily conserved *in vivo* molecular responses to gliotoxin exposure and perhaps toxicity.

The exposure of mammalian cells to gliotoxin increases the levels of reactive oxygen species (ROS) [62], and deletion of yeast genes responsible for dealing with oxidative stress causes cells to be sensitized to gliotoxin [15].

However, the relationship between gliotoxin and oxidative stress induction is complicated. In addition to the aforementioned relationship between GSH and gliotoxin, recent studies in fungi have demonstrated that the presence of gliotoxin can increase survival and growth when *A. fumigatus* is cultured in the presence of high levels of growth-inhibiting H_2O_2 [17]. Moreover, gliotoxin can directly reduce the levels of ROS produced in *A. fumigatus* when exposed to H_2O_2, suggesting that under appropriate conditions gliotoxin can exert antioxidant activities [63]. While the mechanism of such activity remains to be elucidated, it is clear from proteomic studies that gliotoxin exposure, either alone or in combination with H_2O_2, can have far-reaching effects on a variety of central metabolic processes such as amino acid and purine metabolism in fungi [63]. It is not inconceivable that these global changes in the fungal proteome each provide a systems-level contribution to alleviating oxidative stress induced by H_2O_2 exposure.

Gliotoxin impact on animal cells

Gliotoxin and related ETPs directly inactivate multiple enzymes and proteins in animal cells, including nuclear factor-κB (NF-κB), NADPH oxidase, and glutaredoxin, by conjugation to thiol groups [64–66]. It has been conclusively demonstrated that gliotoxin is taken up by animal cells in a GSH-dependent manner whereby this normally protective molecular species facilitates the concentration of gliotoxin within animal cells [59]. Specifically, upon uptake intracellular gliotoxin is reduced to the dithiol form, resulting in GSH depletion and concomitant formation of GSSG. Apoptosis ensues, followed by oxidation of dithiol gliotoxin to reform gliotoxin [59]. Gliotoxin then effluxes from the cell in a pseudocatalytic manner and may be taken up by adjacent cells to continue its cytocidal effects. In addition to having an essential role in gliotoxin biosynthesis as a sulfur donor [25,26], GSH has also been shown to have an important role in mediating gliotoxin-induced cytotoxicity in animal cells and fungi. Interestingly, diminution of intracellular GSH levels using L-buthionine-sulfoxamine (BSO), a specific inhibitor of GSH synthesis, significantly attenuated the cytotoxic effect of gliotoxin towards human neuroblastoma SH-SY5Y cells, possibly due to a deficit in GSH-mediated cleavage of the disulfide bridge of gliotoxin to produce reactive dithiol gliotoxin [58]. This study highlighted the seemingly contradictory effects of antioxidants in enhancing cellular sensitivity to disulfide compounds.

Peroxiredoxins (Prxs) constitute a ubiquitous family of antioxidant enzymes that catalyze the reduction of peroxides. This is achieved with two electrons derived from NADPH via an electron-conveying system comprising thioredoxin and thioredoxin reductase. Nanomolar levels of gliotoxin can protect against oxidative damage by converting H_2O_2 to water in HeLa cells, where it functions to accept electrons from NADPH in the thioredoxin redox system [6]. Gliotoxin could, in turn, attenuate H_2O_2-induced angiogenesis in a dose-dependent manner, thereby acting as an anti-angiogenic agent. This anti-angiogenic effect of *A. fumigatus* was completely attenuated by deletion of *laeA*, the global regulator of secondary metabolism [67]. These studies also revealed that gliotoxin was responsible for approximately 50% of this anti-angiogenic activity because culture extracts from *A. fumigatus ΔgliP*, deficient in gliotoxin biosynthesis only, did not abolish angiogenesis. In murine melanoma cells, Prx II silencing enhanced lung metastasis *in vivo*. Gliotoxin, owing to its ability to exert a Prx-like activity, inhibited the proliferation and migration as well as lung metastasis of Prx II-deficient melanoma cells. This *in vivo* study implicated the Prx II mimetic ability of gliotoxin as a promising therapeutic drug for preventing melanoma metastasis [68].

Further studies have dissected the molecular basis and investigated applications of ETP-mediated anti-angiogenesis. Tumor cells must adapt to a hypoxic environment to survive and grow. This is mediated by hypoxia-inducible factor (HIF-1), whereby the O_2-regulated HIF-1α subunit of the HIF-1 transcription factor is required for activation of multiple genes involved in tumor progression and angiogenesis [69]. Under hypoxic conditions, HIF-1α undergoes nuclear translocation where it interacts with HIF-1β and binds to target DNA sequences. Upon recruitment of additional proteins (e.g., p300), gene expression is activated to mount an adaptive response to hypoxia [70]. It has been speculated that disruption of this hypoxic response could form an effective anticancer strategy. Interestingly, gliotoxin and the ETPs chaetocin and chetomin can inhibit the HIF-1α–p300 interaction by ejecting an essential Zn^{2+} cofactor from p300 via the formation of a $Zn^{2+}(ETP)_2$ moiety [71]. Moreover, exposure to gliotoxin resulted in anti-proliferative effects on cultured tumor cells, which were reversible by Zn^{2+} supplementation. Furthermore, Zn^{2+} can protect HepG2 cells against toxicity of the ETP sporidesmin from *Pithomyces chartarum* which causes facial eczema in sheep [72]. Indeed, Zn^{2+} supplementation of ruminant diets is an effective prophylactic treatment for this disease [73]; it is tempting to speculate that this could be effected via disruption of the HIF-1α–p300 adaptive response. More recently it has been shown that gliotoxin, chaetocin, and chetomin could significantly decrease tumor growth in a prostate cancer xenograft model system [69]. Although gliotoxin exposure did not impact on the expression of HIF-1α regulated genes, these authors speculated that the antitumor activity of gliotoxin could be mediated via farnesyltransferase inhibition because inhibition of this enzyme can attenuate angiogenesis by interfering with endothelial cell migration [74].

As described in Figure 4, gliotoxin induces apoptotic cell death by activating the pore-forming proapoptotic Bcl-2 family member Bak to elicit ROS generation, the mitochondrial release of apoptogenic factors, and caspase-3 activation. Thus, not only has this work elucidated the mechanism of gliotoxin-induced apoptosis but also it has uncovered the molecular mechanisms of Bcl-2 family initiation [16,75]. It is possible that induction of gliotoxin-dependent apoptosis could impact on the severity and course of *Aspergillus* infection by destroying lung epithelium or by killing alveolar macrophages [62]. NF-κB is a ubiquitously expressed proinflammatory transcription factor composed of different combinations of members of the Rel family of proteins. As shown in Figure 4 gliotoxin has been well-characterized as an inhibitor of NF-κB activation [64], and this then led to a plethora of studies which utilize gliotoxin as a specific NF-κB inhibitor [76–78]. Gliotoxin

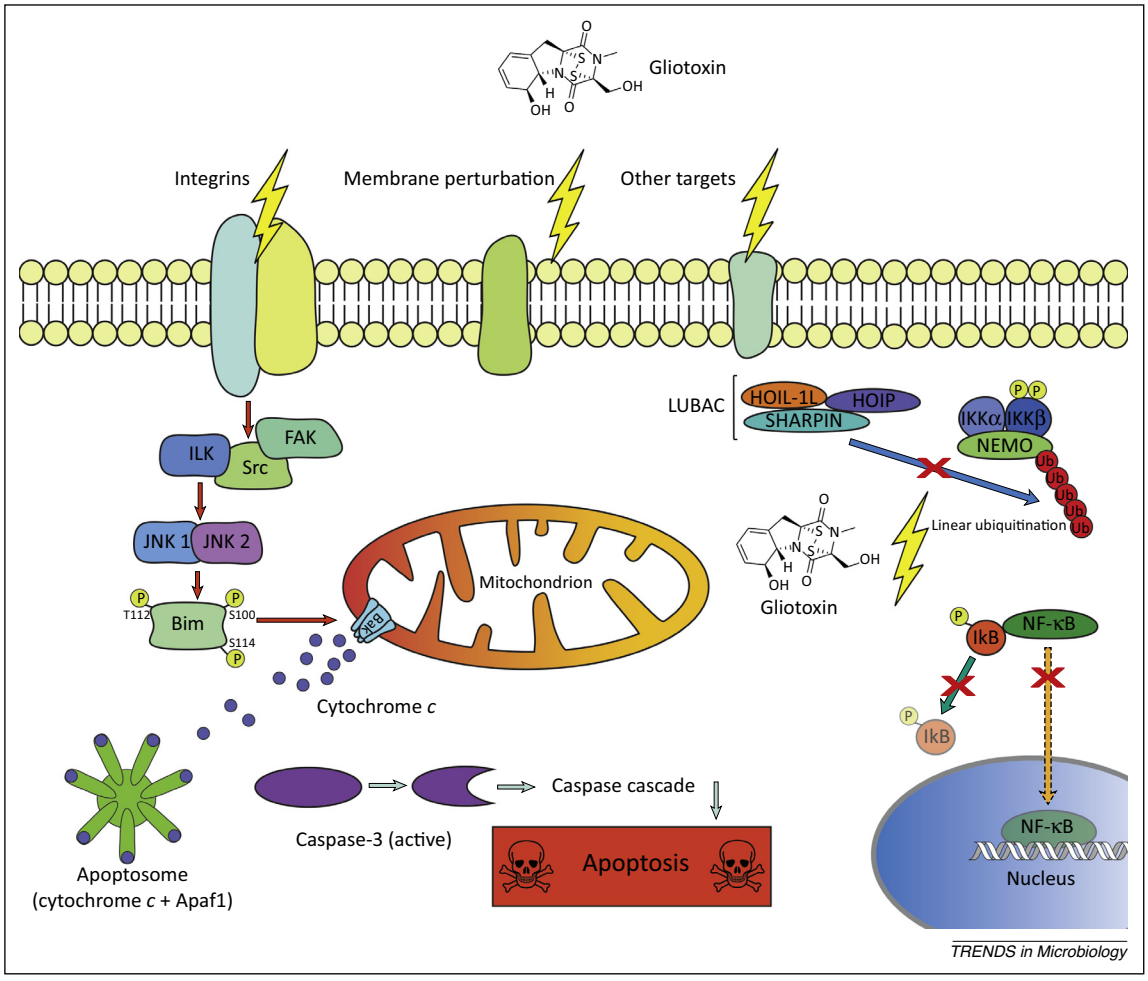

Figure 4. Gliotoxin induction of apoptosis in mammalian cells through various specific cellular targets. Upstream of the pore-forming proapoptotic Bcl-2 family member Bak, gliotoxin-mediated apoptosis requires the c-Jun N-terminal kinase 1 and 2 (JNK1/2)-mediated phosphorylation (P) of the BH3-only protein BimEL at three sites (S100, T112, and S114). This triple phosphorylation increases the stability of BimEL, increases its binding affinity for Bcl-2-like survival factors, and activates Bak more effectively. Notably, triple-mutated Bim is unable to transduce the gliotoxin–JNK-initiated apoptotic stimulus to Bak. Compared to wild type, mice lacking Bak were significantly less susceptible to *A. fumigatus* infection, which demonstrates the *in vivo* relevance of this gliotoxin-induced apoptotic pathway [16,62,75]. In resting cells, NF-κB is localized to the cytoplasm because of binding to inhibitory protein IkB. Upon activation, NF-κB inducing kinase (NIK) is activated, which in turn activates a complex of specific IkB kinases (IKKs) resulting in IkB phosphorylation. Phosphorylation of IkB leads to a rapid ubiquitination (Ub) which makes it a substrate for the proteasome. The active NF-κB complex then translocates to the nucleus and initiates the expression of target genes. Gliotoxin inhibits NF-κB activation by preventing IkB degradation [64]. Gliotoxin also suppresses NF-κB activation by inhibiting the linear ubiquitin (Ub) chain assembly complex (LUBAC). This is the first selective small-molecule inhibitor of this complex to be identified [91]. Abbreviations: Apaf1, apoptotic protease activating factor 1; FAK, focal adhesion kinase; HOIL-1L, heme-oxidized IRP2 ubiquitin ligase-1; HOIP, HOIL-1L interacting protein; ILK, integrin-linked kinase; NEMO, NF-κB essential modulator; SHARPIN, SHANK-associated RH domain interacting protein; Src, Sarcoma family nonreceptor protein tyrosine kinase.

has also been utilized to demonstrate that NF-κB inhibition reverses tumor necrosis factor-α (TNF-α)-induced eosinophil survival, highlighting the major role of NF-κB in TNF-α-induced inhibition of eosinophil apoptosis [79]. It is clear from these selected studies that gliotoxin is a potent small molecule capable of targeting and revealing multiple aspects of animal cell biochemistry.

Concluding remarks

Our knowledge of gliotoxin biosynthesis, functionality, and utility has increased dramatically over recent years [80]. Specifically, it is now clear that gliotoxin biosynthesis (i) involves novel enzymology, (ii) unusually deploys GSH as a substrate for biosynthetic intermediate sulfurization, (iii) has resulted in the evolution of a self-protection system mediated by GliT, (iv) results in the production of a suite of additional related metabolites (some with diagnostic potential [38,81]), and (v) is post-biosynthetically negatively regulated by GtmA activity. Reconsidering the established view of gliotoxin as a 'toxin', it is becoming increasingly clear that its antioxidant and signaling roles [82] need further evaluation and study. Whether this antioxidant ability is related to the influence that gliotoxin, or pathway components, exert on the biosynthesis of unrelated metabolites in *A. fumigatus* remains to be established (Box 1). GSH-mediated reduction of gliotoxin in animal cells and

Box 1. Outstanding questions

- What are the roles of *gliH* and *gliM* in gliotoxin biosynthesis?
- What are the interactions and roles of the many regulatory genes which appear to control gliotoxin biosynthesis?
- What is the mechanism of independent regulation of *gliT* expression?
- How can the interplay between gliotoxin and other natural product biosynthetic pathways in *A. fumigatus* be elucidated?
- What is the redox systems significance of the interplay between GSH, EGT, and gliotoxin in fungi?
- Is there an anticancer role of gliotoxin and related ETPs? Can its role as a probe of cellular systems in animal cells be further developed?

fungi appears to be central to the potency of the metabolite to alter and impair cellular systems. In the future, contextualization of gliotoxin effects on eukaryotic cells may benefit from cognizance of its multiple actions and reactivity of dithiol gliotoxin. Moreover, in fungi capable of gliotoxin biosynthesis, we now know that exogenous gliotoxin induces *gli* cluster expression and concomitant gliotoxin biosynthesis [21,54]; the impacts and utility of these observations cannot be underestimated.

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