The secondary fungal metabolite gliotoxin targets proteolytic activities of the proteasome

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Background: The fungal epipolythiodioxopiperazine metabolite gliotoxin has a variety of toxic effects such as suppression of antigen processing, induction of macrophagocytic apoptosis and inhibition of transcription factor NF-κB activation. How gliotoxin acts remains poorly understood except that the molecule's characteristic disulfide bridge is important for immunomodulation. As this fungal metabolite stabilizes the NF-κB inhibitor IκBα in the cytoplasm, we decided to investigate its molecular mechanism of action.

Results: We show that gliotoxin is an efficient, noncompetitive inhibitor of the chymotrypsin-like activity of the 20S proteasome *in vitro*. Proteasome inhibition can be reversed by dithiothreitol, which reduces gliotoxin to the dithiol compound. In intact cells, gliotoxin inhibits NF-κB induction through inhibition of proteasome-mediated degradation of IκBα.

Conclusions: Gliotoxin targets catalytic activities of the proteasome efficiently. Inhibition by gliotoxin may be countered by reducing agents, which are able to inactivate the disulfide bridge responsible for the inhibitory capacity of gliotoxin.

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Introduction

Invasive fungal infections are among the most serious problems in immunocompromised patients, causing elevated mortality rates [1–2]. The precise molecular mechanisms of many fungal pathogeneses are unclear, but one possible agent is the secondary fungal metabolite gliotoxin that contributes to the immunosupressive and other toxic effects of infections by opportunistic pathogens including *Aspergillus fumigatus* and *Candida albicans* [3–5]. Gliotoxin and related compounds can be life-threatening for immunocompromised individuals such as AIDS patients suffering from disseminated aspergillosis. Gliotoxin has a variety of antimicrobial effects [3] and induces apoptosis in different cell types such as macrophages, thymocytes, spleen cells and cells of the mesenteric lymph nodes [2–6].

Gliotoxin and related molecules are members of the fungal epipolythiodioxopiperazine toxins, a compound class characterized by a heterobicyclic core containing a polysulfide bridge (2–4 atoms of sulfur) [3–7]. The sulfide bridge plays a crucial role in the toxic effect, because its chemical elimination abolishes inhibition of virus proliferation and bacterial or eukaryotic cell proliferation [8]. It is known that low molecular weight thiols may protect cells against the toxic effects of gliotoxin. Several studies suggested a potential interaction involving the formation of mixed disulfide bonds between the toxin's sulfide groups and the disulfide or thiol groups of cellular proteins to explain the mechanism of gliotoxin action [7–10].

Pahl *et al.* [11] reported that gliotoxin is a potent inhibitor of transcription factor nuclear factor κB (NF-κB) activation in T and B cells induced in response to a variety of stimuli. Heterodimers of p50/p65 are the most abundant members of the Rel/NF-κB family of transcription factors that controls the expression of a wide variety of genes implicated in immune responses, inflammation and cellular proliferation. Dysregulation of NF-κB is associated with cellular transformation and the maintenance of a high anti-apoptotic threshold in transformed cells. NF-κB activity is regulated by its sequestration in the cytoplasm by the inhibitor of κB α (IκBα), which is susceptible to proteasome-mediated degradation [12,13]. Following cell activation, IκBα is phosphorylated on serine residues 32 and 36 [14–18], and ubiquitinated on lysine residues 21, 22, and the accessory residues 38 and 47 [19–21]. Ubiquitination of IκBα is achieved by an ubiquitin-conjugating activity possibly consisting of several different enzymes [22] working with an E3 enzyme complex of the SCF-type (for Skp1-Cullin-F-box-containing protein complex [23]), the receptor component of which is the F-box protein family member β-transducin-repeat-containing protein (βTrCP) [24–27].

Through a mechanism still under investigation, IκBα is then targeted for degradation by the proteasome [19–21]. The multicatalytic proteolytic complex generally referred to as proteasome occurs in several forms in eukaryotic cells [28] — the 26S proteasome or the 20S proteasome that may associate or not with modulatory subunits. Proteasomes are pivotal in the control of the cell cycle, cell division and differentiation, development, DNA repair, regulation of immune and inflammatory responses, apoptosis, antigen presentation, modulation of the expression of cell surface receptors, and transcription of genes requiring participation of certain transcription factors such as NF-κB and activating protein 1 (AP-1) [28].

The 20S proteasome is a high molecular mass (700 kDa) protease present in the cytoplasm and nucleus of eukaryotic cells and represents up to 1% of total cellular protein [29–31]. It is composed of four stacked rings, each of which carries seven subunits of 21–35 kDa, the inner rings containing the β subunits and the outer rings the α subunits [32]. The rings of the eukaryotic proteasome are composed of up to 14 different subunits that have at least three different catalytic activities, the trypsin-like, chymotrypsin-like and peptidylglutamyl-peptide hydrolase activities, localizing to three active sites [33,34]. The cleavage of peptide bonds at the carboxyl end of branched-chain amino acids and between short-chain neutral amino acids has also been described [35].

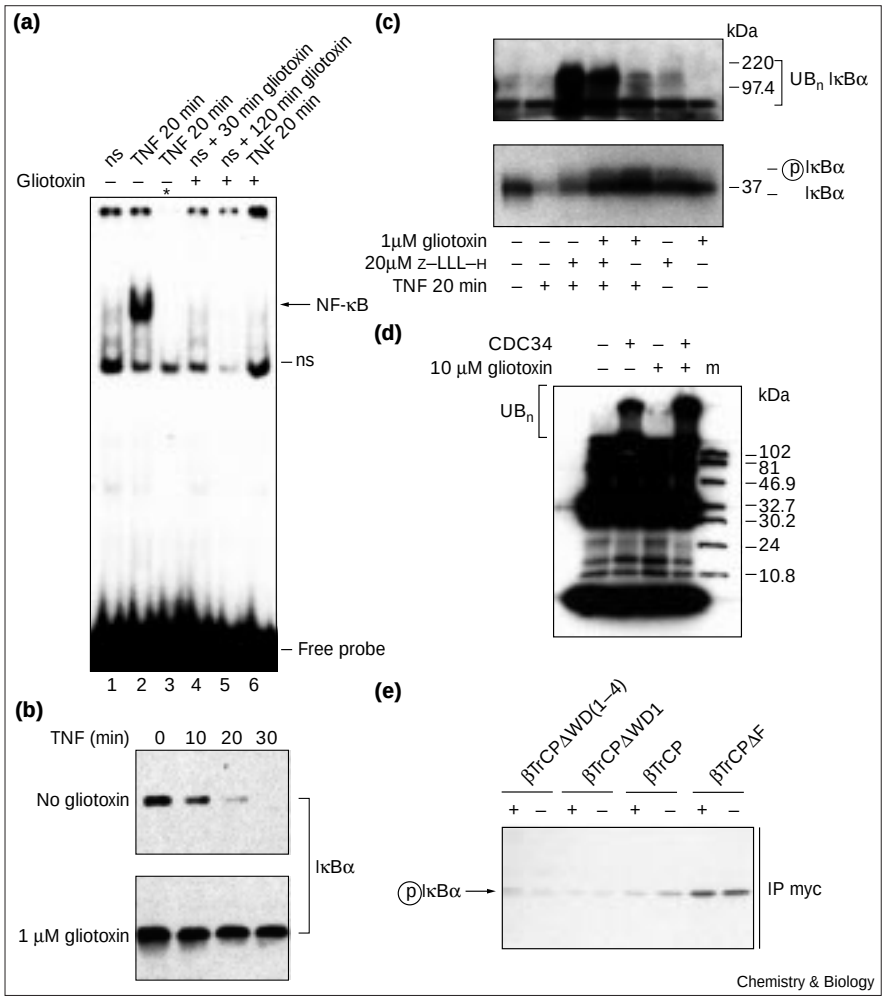
Because of the central role of proteasomes in the activation of NF-κB, we decided to study the possibility that gliotoxin has an inhibitory effect on the proteolytic activities of proteasomes. We show that gliotoxin inhibits the degradation of the succinylated fluorogenic peptide leucine–leucine–valine–tyrosine–amidomethylcoumarin (Suc–LLVY–amc), which is cleaved by the chymotrypsin-like activity of the proteasome in a noncompetitive manner, but does not affect the proteases trypsin, chymotrypsin and calpain. We also present evidence that gliotoxin administration precludes IκBα degradation *in vitro* and in cell culture and is therefore responsible for suppression of NF-κB activation.

Results and discussion

Gliotoxin inhibits NF-κB activation by stabilizing IκBα

Administration of low doses of gliotoxin is sufficient to prevent tumor necrosis factor (TNF)-induced NF-κB activation in HeLa cells or peripheral blood mononuclear cells. Gliotoxin (1 μM) was added to the culture medium of HeLa cells. Following stimulation by TNF, the cytoplasm and the nucleoplasm were fractionated, and nuclear extracts were analysed using an electrophoretic mobility shift assay (EMSA; Figure 1a). Results show that a 30 minute exposure to gliotoxin prevents NF-κB binding beyond its baseline level constitutively present in HeLa cells, and after 2 hours of preincubation, gliotoxin is even able to down-regulate the constitutive level of NF-κB binding (Figure 1a, compare lanes 1, 4 and 5), presumably by inhibiting at least part of the basal turnover of IκBα. When cells were exposed to gliotoxin, TNF stimulation was no longer able to provoke significant NF-κB binding (compare lanes 2 and 6). Accordingly, a stabilization of IκBα in cytoplasmic extracts of gliotoxin-treated cells is observed following TNF stimulation (Figure 1b). To ascertain that down-regulation of IκBα proteolysis is really caused by proteasome inhibition and not by interference with signal transduction, we tested whether gliotoxin could prevent the accumulation of ubiquitinated forms of IκBα or prevent its binding to the receptor component of the E3 SCF-type ubiquitin ligase, βTrCP [24–27]. For this purpose, HeLa cells, treated or not with TNF in the presence and absence of gliotoxin and/or the proteasome inhibitor Z–LLL–H (also called MG132), were lysed in a buffer containing the alkylating agent iodoacetamide (10 mM) that blocks ubiquitin carboxy-terminal hydrolases and isopeptidases. In immunoblots, phosphorylated and ubiquitinated IκBα could be detected when cells had been treated with 1 μM of gliotoxin and/or 20 μM of Z–LLL–H, and stimulated with TNF for 20 minutes (Figure 1c). In addition, we carried out an *in vitro* test of the ability of gliotoxin to interfere with ubiquitin-transfer reactions from yeast E1 to recombinant E2 CDC34. As can be seen from Figure 1d, 10 μM gliotoxin does not inhibit ubiquitin transfer in an E1/E2 *in vitro* system containing yeast E1, CDC34 and radiolabeled glutathione-S-transferase (GST)–ubiquitin. Moreover, phospho-IκBα was still able to bind to, and coprecipitate with, βTrCP and βTrCPΔF, an F-box deletion mutant of βTrCP [27], in the presence of 1 μM gliotoxin (Figure 1e). As the ubiquitinated forms of IκBα observed in the presence of gliotoxin depend on the preceding phosphorylation of serine residues 32 and 36, we conclude that gliotoxin does not significantly interfere with IκBα phosphorylation, which is in agreement with the findings described previously [11]. Taken together, these results indicate that gliotoxin does not significantly interfere with signal-induced modifications of IκBα, but merely with its proteasome-mediated degradation, as suggested by our *in vitro* experiments. Previously, we showed that the amino and carboxyl termini of IκBα, with the carboxyl terminus being the noninducible part of the signal responsible for determining susceptibility to degradation by the catalytic core of the proteasome [36]. The suppression of NF-κB activation may therefore be caused by the inhibition of proteasome-resident catalytic activities by gliotoxin. To further confirm this hypothesis, we examined four other known proteasome substrates, p53 [37], β-catenin [38], dihydrofolate reductase (DHFR) [39] and ornithine decarboxylase (ODC) [40], whose regulatory pathways leading to proteasome-mediated degradation are different from that of IκBα. ODC is a ubiquitin-independent proteasome substrate.

Figure 1



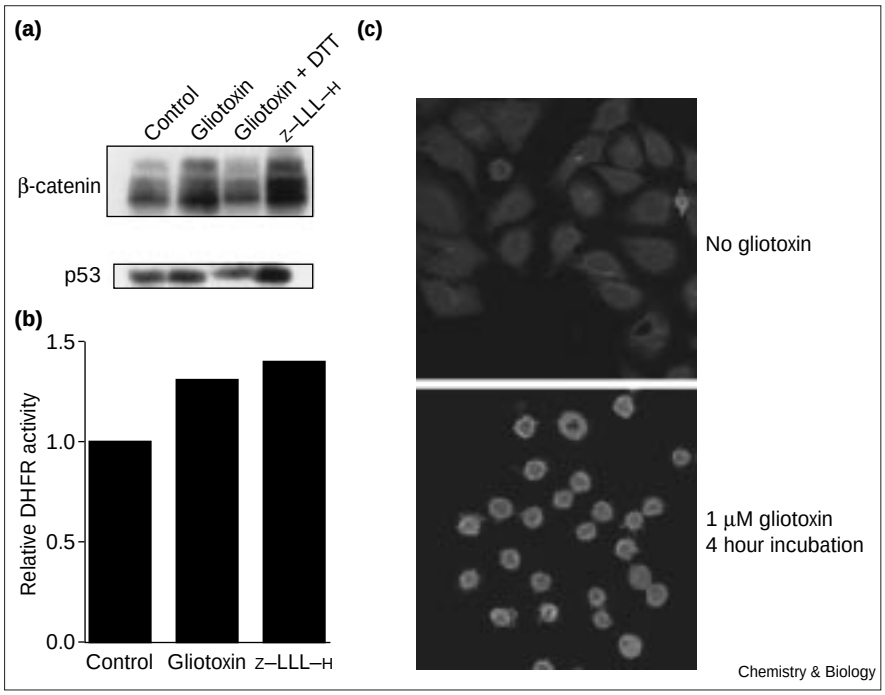
Gliotoxin inhibits NF-κB activation in intact cells by stabilizing phosphorylated and ubiquitinated IκBα. **(a)** Down-regulation of NF-κB activation by gliotoxin. HeLa cells were exposed to 1 μM gliotoxin where indicated, stimulated with TNF or left untreated during the indicated time periods, lysed, fractionated and subjected to an electrophoretic mobility shift assay. Nuclear extracts (4 μg) were probed with a ^{32}P-labeled oligonucleotide containing six consecutive binding sites for NF-κB (κB elements) taken from the long terminal repeat of HIV-1. Lanes 1–3, no gliotoxin; lanes 4–6, 1 μM gliotoxin. Lane 3, in the absence of gliotoxin and in the presence of the unlabeled, added in excess over the radioactive, probe (indicated by an asterisk). ns, nonspecific signal. **(b)** Stabilization of IκBα by gliotoxin. HeLa cells were exposed to 1 μM gliotoxin (bottom) or not (top) and lysed after TNF stimulation. Cytoplasmic extracts were fractionated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and blotted onto nitrocellulose. IκBα was detected by monoclonal antibody 10B. **(c)** Insignificant interference of gliotoxin with IκBα signal-induced modification. Detection of ubiquitinated (top panel) and phosphorylated (bottom panel) forms of IκBα in the presence of gliotoxin. Exposures were taken from the same gel. HEK293 cells were left untreated or preincubated for 30 min with 1 μM gliotoxin and/or 20 μM proteasome inhibitor Z–LLL–H (MG132), and stimulated with TNF for 20 min, where indicated. Cytoplasmic extracts were prepared by immediate lysis in the presence of 10 mM of iodoacetamide, blotted and immunodetected with a polyclonal anti-IκBα antibody directed against the carboxyl terminus. **(d)** Lack of gliotoxin-mediated inhibition of ubiquitin-transfer reactions in an E1/E2 *in vitro* system. Yeast E1 and CDC34 enzymes were incubated with radiolabeled GST–ubiquitin fusion proteins in a buffer containing $MgCl_2$ and ATP. Reactions were incubated at 37°C for 30 min, fractionated using SDS–PAGE, and analysed by autoradiography. m, molecular weight markers. **(e)** Detection of S32/36-phosphorylated IκBα specifically co-precipitated with over-expressed, myc-tagged βTrCP proteins. Cells of the 293 human embryo kidney cell line were transfected with a pcDNA3.1-βTrCP-myc construct [27] and, 24 h after transfection, pretreated with 1 μM gliotoxin for 30 min and stimulated with TNF for 20 min (+) or left unstimulated (–). Extracts were subjected to immunoprecipitation using coupled anti-myc-protein G-sepharose conjugates, blotted, and immunodetected using anti-phospho-IκBα antibody. βTrCPΔWD(1–4) and βTrCPΔWD1 are βTrCP deletion mutants incapable of associating with phospho-IκBα (M.K., unpublished observations) and show the background signal not resulting from specific binding.

Exposure of interleukin-2-activated peripheral blood mononuclear cells to 10 μM gliotoxin stabilized these substrates over a period of 4 hours, albeit to a lesser extent than 10 μM Z–LLL–H (Figure 2a, compare lanes 1, 2 and 4 for p53 and β-catenin). The effect of gliotoxin could be reversed by addition of the reducing agent dithiothreitol (DTT; 1 μM) to the culture medium (Figure 2a, compare lanes 1, 2 and 3). Equally, gliotoxin administration elicited the accumulation of DHFR (Figure 2b) by a factor of 1.3, as measured by an enzymatic assay, and of ODC (Figure 2c) by a factor of 6.6, as measured by semiquantitative image analysis using a scanning confocal microscope (in control reactions, there was no measurable fluorescence light emission from gliotoxin-treated or gliotoxin-untreated cells when the ODC antibody was replaced by a nonspecific control antibody).

Gliotoxin affects primarily the chymotrypsin-like peptidase activity of the 20S proteasome

We examined the effect of two different gliotoxin concentrations (40 μM and 100 μM) on the degradation of three different fluorogenic peptides: Suc–LLVY–amc, LSTR–amc, and LLE–na, which are targets of the different proteolytic activities of proteasomes (see the

Figure 2



Gliotoxin elicits the accumulation of proteasome substrates in cell culture. Peripheral blood mononuclear cells were exposed to 10 μM gliotoxin or 20 μM z–LLL–H where indicated, or left untreated. After 4 h of exposure to gliotoxin, gliotoxin + 1 mM DTT or z–LLL–H, cells were lysed, and (a) β-catenin or p53 proteasome substrates were immunodetected using specific antibodies. (b) Dihydrofolate reductase (DHFR) assays were carried out as described in the Materials and methods section. (c) Accumulation of ornithine decarboxylase in gliotoxin-exposed, as compared with untreated, cells was performed by immunological detection of the protein.

Materials and methods section). Degradation of the fluorogenic peptide Suc–LLVY–amc was considerably inhibited at both concentrations of gliotoxin (Table 1) with an *in vitro* IC_{50} value of approximately 10 μM (Figure 3a), whereas the other catalytic activities of the proteasome were inhibited to a lesser extent (Table 1). Moreover, after treatment with 1 μM of gliotoxin, a 63% inhibition of LLVY–amc-hydrolyzing activity could be observed in crude cell extracts of peripheral blood lymphocytes. In addition, we isolated proteasomes from gliotoxin-treated and gliotoxin-untreated cells from two different cell lines (see the Materials and methods section). Isolated proteasomes were tested for their ability to hydrolyze suc–LLVY–amc and LLE–na. A 61% inhibition of LLVY-hydrolyzing activity was observed in HEK-293 proteasomes and a 66% inhibition of this activity was observed in BHK-21 proteasomes when cells had been treated with 1 μM gliotoxin. *In vitro* addition of 50 μM z–LLL–H to extracts from gliotoxin-untreated cells produced a 96% inhibition in LLVY-hydrolyzing activity and a similar inhibition of LLE-hydrolyzing activity, indicating that the isolated proteases were almost 100% proteasomes.

Table 1

Effect of gliotoxin on the different proteolytic activities of purified proteasomes.

		Inhibition (%)	
Catalytic activity	Fluorogenic peptide	100 μM gliotoxin	40 μM gliotoxin
Chymotrypsin-like	LLVY–amc	95	72
Trypsin-like	LSTR–amc	37	39
Peptidylglutamyl hydrolase	LLE–na	34	6

Proteolytic activities were measured with fluorogenic peptides as described in the Materials and methods section. 5 μg of 20S proteasome were incubated for 20 min at 37°C with gliotoxin at final concentrations of 40 μM or 100 μM and one of the listed fluorogenic peptides at the indicated concentrations. Activities are expressed relative to no gliotoxin treatment (control), equal to 0% inhibition. Indicated values are means of three experiments.

To verify whether gliotoxin inhibits proteolytic activities closely related to the proteasome, we tested its effect on the activity of the proteases trypsin, chymotrypsin and calpain at two different concentrations, 10 μM and 100 μM. Gliotoxin down-regulated the chymotrypsin-mediated proteolysis of Suc–LLVY–amc by about 12% at 100 μM concentration. No effect was observed for trypsin. We found ~20% inhibition of calpain proteolytic activity at a gliotoxin concentration of 100 μM. At 10 μM gliotoxin, the inhibition observed for all three proteases was less than 5%. In conclusion, at concentrations used for rather efficient proteasome inactivation, gliotoxin does not significantly inhibit trypsin, chymotrypsin or calpain. Thus the inhibition affects proteasomes but not proteases related to the proteolytic activities of the proteasome.

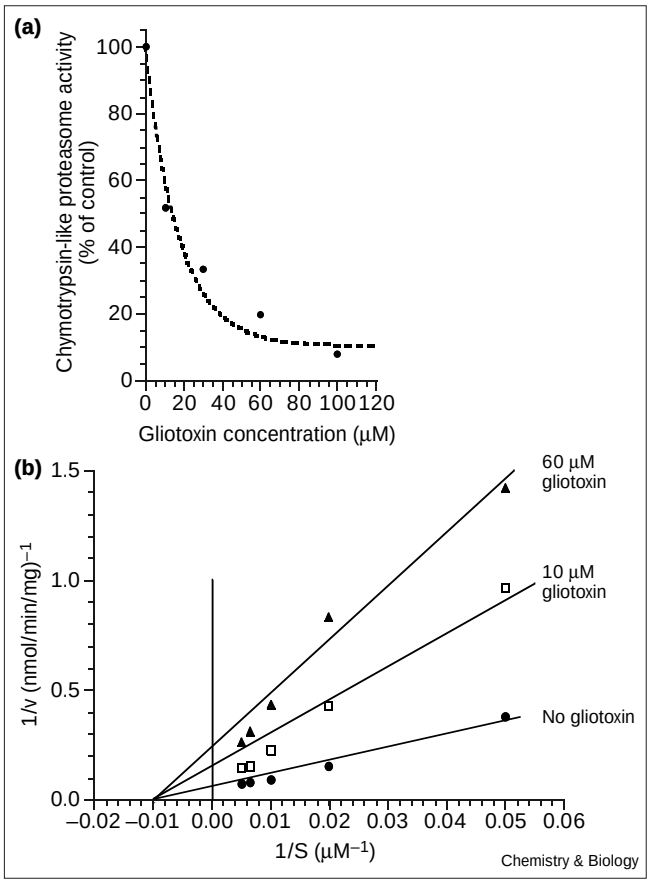
Gliotoxin acts as a noncompetitive inhibitor of the 20S proteasome

Gliotoxin inhibited proteolysis of Suc–LLVY–amc, as shown by the characteristic pattern of the Lineweaver–Burk plots (Figure 3b). Measurements were taken with a no-gliotoxin control and two different concentrations of gliotoxin (10 and 60 μM), and linear regression analysis was performed on the experimental data. The diminution of the V_{max} of the proteasomal chymotrypsin-like activity with increasing drug concentration, together with the constant K_m, demonstrate that gliotoxin is a noncompetitive inhibitor for the degradation of Suc–LLVY–amc by the 20S proteasome.

As the disulfide bridge of gliotoxin has been shown to be essential for the majority of its physiological effects, we decided to examine whether it was also involved in proteasome inhibition. Incubation of purified 20S proteasomes with DTT suppresses the effect of gliotoxin on the degradation of Suc–LLVY–amc in a dose-dependent manner. In the absence of DTT, 40 μM gliotoxin inhibited degradation of this peptide by nearly 70%. DTT (5 mM) reduced the effect of gliotoxin to less than half, and 10 mM DTT completely abolished its inhibition of proteasome activity (Figure 4a). When gliotoxin was preincubated with the 20S proteasome, addition of 10 mM DTT could fully restore proteasome Suc–LLVY-hydrolyzing activity (Figure 4b), indicating that inhibition of the 20S proteasome by gliotoxin is a reversible phenomenon. The chemical dissociation of the disulfide bridge was achieved by preincubating gliotoxin with DTT because low molecular weight thiols are able to reduce gliotoxin to the dithiol compound. The latter compound but not the bis(methyl)thio form may be re-oxidized to gliotoxin (Figure 4c) [3]. On the contrary, glutathione (reduced form GSH), used at the same concentration as DTT, was unable to abolish the gliotoxin effect on proteasomes (Figure 4a). Consistent with the reversibility of gliotoxin-mediated proteasome inhibition observed in our experiments, incubation of gliotoxin-treated HeLa cells with 1 mM of DTT in the culture medium partially restores TNF-induced NF-κB binding to its consensus sequence (Figure 4d). Furthermore, to test whether intracellular glutathione levels, which are in the range of 1–10 mM [41], interfere with the inhibitory capacity of gliotoxin, we performed a dose–response experiment for 1, 5 and 10 mM glutathione or DTT in comparison (Figure 4e). Reduced glutathione is not able to reverse the inhibitory effect of gliotoxin on IkBα proteolysis, which is in contrast to DTT. We conclude that the inhibitory effect of gliotoxin on the proteasome depends on an intact disulfide bridge of the molecule.

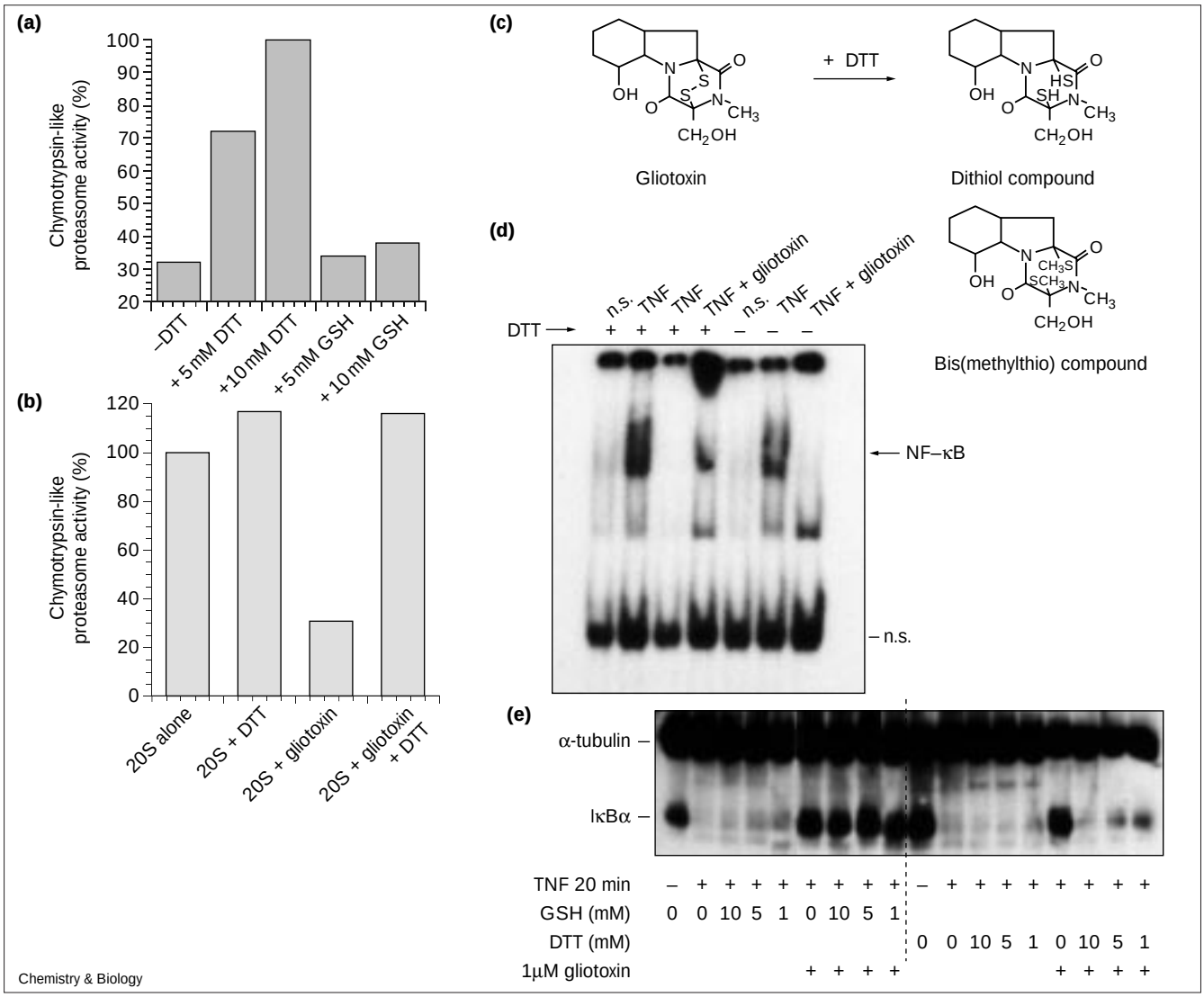
Gliotoxin has drawn considerable attention because of its involvement in fungal diseases, especially in immunocompromised patients. Prior attempts to study its molecular mechanism of action hinted at the importance of the molecule's disulfide bridge but failed to yield a satisfactory explanation for such diverse effects as suppression of antigen processing, induction of apoptosis and suppression of NF-κB activation in stimulated cells. Our results demonstrate that gliotoxin is an inhibitor of 20S proteasomes and, as such, inhibits the degradation of Suc–LLVY–amc, which is a target for the proteasomal chymotrypsin-like activity. The drug acts as a noncompetitive proteasome inhibitor, as shown by the characteristic pattern of the Lineweaver–Burk plot. Gliotoxin may target the proteasome by reversible covalent modification involving mixed disulfide bonds at or near the active site of the chymotrypsin-like activity. The slight inhibition of

Figure 3



Gliotoxin inhibits the proteasome *in vitro*. (a) Quantitative effect of gliotoxin on the hydrolysis of Suc–LLVY–amc by the 20S proteasome. Biochemically purified 20S proteasome (5 μg) was incubated for 20 min at 37°C with the indicated concentrations gliotoxin and 80 μM of Suc–LLVY–amc. Proteasome activity was measured as described in the Materials and methods section. (b) Lineweaver–Burk analysis of gliotoxin-mediated inhibition of Suc–LLVY–amc hydrolysis. 20S proteasome (5 μg) was incubated at 37°C with different concentrations of Suc–LLVY–amc. ●, in the absence of gliotoxin; □, in the presence of 10 μM or, ▲, 60 μM of gliotoxin. Activities were measured as described in the Materials and methods section, and linear regression analysis was applied to experimental data.

Figure 4



Gliotoxin-mediated proteasome and NF-κB inhibition depend on an intact bicyclic structure featuring the disulfide bridge. (a) Gliotoxin-mediated inhibition of the proteasome chymotrypsin-like activity after preincubation of gliotoxin with or without DTT or glutathione at 5 mM or 10 mM concentration. Pretreated gliotoxin was incubated with 5 μg 20S proteasome to give a final concentration of 40 μM. The degradation of Suc–LLVY–amc was measured as described in the Materials and methods section. Activities are expressed relative to no gliotoxin treatment (control) that equals 100% activity. (b) Reversal of gliotoxin-mediated proteasome inhibition by DTT. Proteasome (5 μg) was preincubated with and without gliotoxin at 40 μM. DTT (5 mM) was added to the preincubation mixture, and the degradation of Suc–LLVY–amc was measured as described in the Materials and methods section. Activities are expressed relative to no gliotoxin treatment (control). (c) Chemical structures of gliotoxin and its dithiol and bisdethiobis(methylthio) modifications. (d) Reversibility of gliotoxin-mediated suppression of NF-κB activation. HeLa cells were incubated with or without 1 mM DTT before TNF stimulation, and gliotoxin was added to 1 μM final concentration where indicated. Electrophoretic mobility shift analysis was carried out as described in Figure 1a. (e) Gliotoxin at 1 μM concentration was preincubated for 10 min at 37°C with or without 1 mM DTT or glutathione (reduced form). Preincubation mixtures were added to HeLa cells. After 30 min, cells were stimulated with TNF for 20 min where indicated and immediately lysed. Equal amounts of extracts were fractionated by SDS–PAGE, blotted and immunodetected with a polyclonal anti-IκBα antibody.

the degradation of the other peptides (Table 1) could also be due to steric hindrance by gliotoxin, which may obstruct substrate access to the catalytic sites. We tested the effect of gliotoxin on chymotrypsin, trypsin, and calpain, and observed no significant inhibitory effect.

Interestingly, the target of gliotoxin, namely the chymotrypsin-like activity of the proteasome, can also be inhibited by amyloid B protein [42] or the antitumor drug aclacinomycin A [43]. The fungal metabolite cyclosporine A, which has immunosuppressive effects, has also

been shown to inhibit this activity and therefore prevent NF-κB activation [44]. Gazos Lopes *et al.* [45] induced apoptosis in Rat-1 and PC12 cells using inhibitors of the chymotrypsin-like activity. This phenomenon was also observed in L1210 leukemia cells [46]. We observed that gliotoxin inhibits NF-κB activation in interleukin-2-pretreated peripheral blood mononuclear cells (data not shown), as other authors have observed in T and B cells [11], and strongly induces apoptosis in many cell types ([6,47–49], and our own observations). Induction of apoptosis mediated by gliotoxin is preceded by phosphorylation of histone H3 [50], but the actual trigger remains unknown. We propose that gliotoxin-mediated inhibition of the proteasomal chymotrypsin-like activity accounts for the drug's pro-apoptotic effect. The latter is in keeping with the drug's suppression of NF-κB activation because NF-κB is known to control the expression of anti-apoptotic proteins [51–53] such as c-IAP1 and c-IAP2 [54,55] or IEX-1L [56]. This gliotoxin-mediated inhibition of the anti-apoptotic program is accompanied by an accumulation of intact p53 (Figure 2a), which favors apoptosis [45]. Furthermore, proteasome mutants in yeast lacking the chymotrypsin-like activity accumulate ubiquitinated proteins when subjected to heat stress or when grown in the presence of canavanine [57,58]. We observe the accumulation of ubiquitinated IκBα when cells are stimulated with TNF in the presence of gliotoxin (Figure 1c). The chymotrypsin-like activity plays a crucial role in substrate degradation by proteasomes and was specifically associated with IκBα degradation [59].

The efficiency of gliotoxin is noteworthy because concentrations as low as 100 nM can affect NF-κB activation in cell cultures [11]. Gliotoxin at 1 μM (Figure 1) can largely prevent NF-κB activation, whereas a concentration of about 20 μM of the inhibitor Z–LLL–H [60] is necessary to reliably produce the same effect [61]. Our results indicate that the disulfide bridge in the bicyclic molecular structure of gliotoxin is necessary for inhibition of proteasome activity, because addition of DTT to gliotoxin-pretreated proteasomes prevented Suc–LLVY–amc proteolysis. However, the dethio compound bisdethiobis(methylthio)gliotoxin (Figure 4c) acts as a platelet-activating-factor antagonist [8], indicating that not all physiological properties of the drug depend on an intact disulfide bridge.

Because of its general toxicity, gliotoxin can only have limited application as a drug. It was extensively studied as an immunosuppressor used in transplantation, with intermediate success [62]. The relatively selective targeting of one particular proteolytic activity of the proteasome, however, may incite further research into the characteristics of this molecule and its interaction with proteasomes.

The secondary fungal metabolite gliotoxin has a multitude of effects, with proteasome inhibition causing many of them. The immunosuppressive property of gliotoxin, which should rely mainly on its inhibition of a specific proteolytic activity of proteasomes, focuses attention on the role of proteasomes as possible targets in several important fungal diseases. Further investigation of the molecular mechanisms of mycotoxin action on proteasome activity should be useful for the development of new drugs in the management of fungal disease.

Significance

The fungal secondary metabolite gliotoxin causes many toxic effects, decimates cattle exposed to this agent, and is life-threatening for immunocompromised patients suffering from disseminated aspergillosis. Gliotoxin induces apoptosis in a number of cell types and suppresses activation of the anti-apoptotic transcription factor NF-κB. We describe the mechanism for many of these effects elicited by gliotoxin, which primarily targets the proteasomal chymotrypsin-like proteolytic activity. Gliotoxin is a highly efficient proteasome inhibitor *in vivo* and *in vitro*. It acts in a noncompetitive way, as shown by Lineweaver–Burk analysis. The disulfide bridge in the heterobicyclic structure of gliotoxin is crucial for the inhibition of the catalytic activity of the proteasome, because destruction of this bridge by the strong reducing agent dithiothreitol reverses the inhibitory effect of the drug, whereas intracellular glutathione concentrations cannot neutralize it.

Although the success of gliotoxin as an immunosuppressive agent in transplantation was limited, gliotoxin may serve as a highly efficient inhibitor of specific proteolytic activities in studies of proteasome-mediated degradation *in vitro* or in cell culture. Further investigation into the precise way gliotoxin exerts its toxic effect on host cell proteasomes may provide useful in developing pharmaceutical agents for the management of aspergillosis.

Secondary metabolites of invasive fungi may very often be considered potential candidates for targeting host cell proteasome catalytic activities, largely accounting for their pathogenicity.

Materials and methods

Reagents and chemicals

Suc–LLVY–amc, N-t-Boc-Leu-Ser-Thr-Arg-7-amido-4-methylcoumarin (LSTR–amc), N-Cbz-Leu-Leu-Glu-β-naphthylamine (LLE–na), 7-amido-4-methylcoumarin (amc), β-naphthylamine (na), rabbit muscle calpain, bovine pancreas α-chymotrypsin, trypsin, β-nicotineamidedihydrogenphosphate (NADPH), dihydrofolate, iodoacetamide, human thrombine, and *Gliocladium fimbriatum* gliotoxin were purchased from Sigma Chemical Company. Recombinant interleukin-2 was from EuroCetus, Netherlands. The short peptide proteasome inhibitor Z–LLL–H (MG132) was synthesized as previously described [60]. Human recombinant TNF was provided by the MRC ADP reagent program. [γ-^{32}P]ATP was purchased from Amersham. Lipofectamine PLUS transfection reagent was from Life Sciences (France). Anti-human IκBα (MAD3) monoclonal antibody 10B [63], polyclonal anti-IκBα antibody

directed against the carboxyl terminus (Santa Cruz), anti-p53 (Santa Cruz), anti-SP1 (Santa Cruz), anti-β-catenin (Transduction Laboratories), and anti-ornithine decarboxylase (Valbiotech) antibodies were used for immunodetection. DHFR activity was measured as described in [64], using dihydrofolate and reduced β-NADPH as (co)substrates.

Cell culture

HeLa cells and cells of the 293 human embryo kidney cell line were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. BHK-21 cells were cultured in BHK-medium from Gibco, supplemented with tryptose broth and 5% fetal calf serum.

Ficoll-purified human peripheral blood mononuclear cells were cultured in RPMI 1640 medium containing 10% fetal calf serum. The latter cells were prestimulated with 20 ng/ml recombinant interleukin-2 for 5 days. Transfections were carried out using Lipofectamine PLUS according to manufacturer's instructions (LifeSciences).

Preparation of cytoplasmic and nuclear extracts by fractionation

Cells were harvested by trypsin treatment, washed with prewarmed phosphate-buffered saline (PBS), and centrifuged. Sedimented cells were gently resuspended and disrupted in 2 ml of lysis buffer containing 0.2% Triton X-100 and protease inhibitors. Where indicated, phosphatase inhibitors and iodoacetamide were added to inhibit phosphatases and isopeptidases. After collecting 0.2 ml to prepare cytoplasmic extracts, 50 ml of lysis buffer was added to the remaining sample which was then centrifuged to sediment nuclei. After careful removal of the supernatant, nuclear pellets were resuspended and maintained with permanent agitation for 1 h at 4°C in 50 μl of a hyperosmotic buffer containing protease inhibitors. Soluble fractions extracted from the nuclear suspension were recovered after centrifugation at 15,000 g for 15 min. Lysis buffer was 50 mM NaCl, 10 mM HEPES pH 8.0, 500 mM sucrose, 1 mM EDTA, 0.5 mM spermidine, 0.15 mM spermine and 0.2% Triton X-100. Hypertonic buffer was 350 mM NaCl, 10 mM HEPES pH 8.0, 25% glycerol, 0.1 mM EDTA, 0.5 mM spermidine and 0.15 mM spermine. Protease inhibitors – 1 mM phenylmethylsulfonyl fluoride, 10 μM leupeptin, 1 μM pepstatin and 50 μM L-1-chloro-3-(4-tosylamido)-4-phenyl-2-butanone – were included in lysis buffers. Absence of nucleoplasmatic contamination from cytoplasmic extracts was checked by detection of SP1 transcription factor.

Electrophoretic mobility shift assays

NF-κB DNA binding assays were performed as described previously [36], and contained 4 μg of nuclear extract incubated for 15 min at room temperature with a [γ-^{32}P]ATP-labeled double-stranded oligonucleotide carrying the κB motif found in the HIV-1 enhancer (5′-ACAAGGGACTTTCCGCTGGGACTTTCCAGGGA-3′). Free DNA was resolved from DNA–protein complexes on a nondenaturing 6% polyacrylamide gel, and positions of radioactive species were determined by autoradiography of the dried gel. Competition experiments were performed by adding a 40-fold molar excess of homologous, unlabeled oligonucleotide to each sample prior to addition of the radiolabeled probe.

Western blot analysis

Aliquots of degradation reactions or cytoplasmic extracts were separated on 10–12% SDS–PAGE gels and transferred onto nitrocellulose membranes (Sartorius) by semi-dry electroblotting. Immobilized antigen-antibody complexes were detected with horseradish peroxidase-conjugated donkey anti-mouse or anti-rabbit immunoglobulins and an enhanced chemiluminescence detection system (ECL, Amersham).

Confocal microscopy

HeLa Cells were grown on microscope slide cover slips, washed twice in ice-cold PBS, fixed with paraformaldehyde and Triton X-100, and subsequently washed three times in PBS. Fixed cells were incubated for 1 h with 2 μg/ml primary antibody (polyclonal anti-ODC antibody from Valbiotech) in PBS/10% FCS/0.1% Tween-20. After five washes in PBS/10% FCS/0.1% Tween-20, cells were incubated with the secondary antibody, an FITC-coupled donkey anti-rabbit antibody. After repetition of the washing procedure, a drop of Vectashield mounting medium was applied to protect the coupled FITC moieties from photobleaching effects. Confocal microscopy was performed on a Leica TCS4d using Leica Scanware to process images. To compare fluorescence intensities of cells exposed to gliotoxin or left untreated, the same settings of the photomultiplier were used. Quantification was carried out using image analysis utilities (Leica).

Immunoprecipitations

For immunoprecipitation experiments, 200 μg of cytoplasmic extract were incubated with anti-myc-agarose conjugates (Santa Cruz) for 60 min at 4°C. Precipitated beads were washed ten times in PBS containing 1% NP-40 and both protease and phosphatase inhibitors. Antibody-antigen complexes were disrupted by boiling in gel loading buffer (Pierce). Precipitated proteins were fractionated by SDS–PAGE and electroblotted onto nitrocellulose membranes.

Purification of proteins

20S proteasome purification to homogeneity. Human placenta was cut into small pieces at 4°C and homogenized in 20 mM Hepes, pH 7.8, 0.1 mM EDTA, 1 mM 2-mercaptoethanol pH 7.8 (4 ml/1 g of tissue) using a Elvehjem potter homogenizer with a Teflon piston. The homogenate was centrifuged at 25,000 g for 2 h at 4°C. The proteasome was purified as previously described [65]. Briefly, the placenta homogenate supernatant was subjected to ammonium sulfate precipitations (35 and 60% saturation). The pellet of the second precipitation was resuspended in 10 mM Tris–HCl pH 7.2, 100 mM KCl, 0.1 mM EDTA, 1 mM 2-mercaptoethanol, and dialysed at 4°C against the same buffer. The first ion-exchange chromatography was performed on a TosoHaas DEAE-5PW column, with a Beckman Gold liquid chromatograph. Purification was achieved in three additional chromatographic steps: ion-exchange on a Mono-Q HR 5/5 column (Pharmacia), gel filtration on a Superose 6 column (Pharmacia), and hydroxyapatite on a HA-1000 column (TosoHaas). Purified proteasome was dialysed against a buffer of 0.1 M Hepes pH 7.8.

Proteasome preparation by ultracentrifugation. Cells were lysed in lysis buffer containing 0.2% Triton X-100. Cell debris was eliminated by centrifugation at 5,000 g for 5 min at 4°C. The supernatant was subjected to ultracentrifugation at 100,000 g for 16 h at 4°C. The pellet was resuspended in 25 mM Tris buffer, pH 7.5.

Protein and peptidase activity assays

Protein concentrations were determined by the bicinchoninic acid assay (Pierce). Assays of 20S proteasome peptidase activities were carried out with fluorogenic peptides. Suc–LLVY–amc was used for chymotrypsin-like activity, LSTR-amc for trypsin-like activity, and LLE–na for peptidylglutamyl-peptide hydrolase activity. The incubation mixture contained 5 μg 20S proteasome, 0.1 M Hepes pH 7.8 with the appropriate peptide substrate concentration in a final volume of 200 μl. Incubation was at 37°C for 20 min, and the reaction was stopped with 300 μl of acid or ethanol depending on the peptide used. After addition of 2 ml distilled water, fluorescence was monitored on a Perkin Elmer LS-5B spectrofluorimeter. The excitation/emission wavelengths were 350 nm/440 nm and 333 nm/410 nm for aminomethylcoumarin and β-naphthylamine reagents, respectively. Calpain activity was measured using succinylated casein according to the QuantiCleave Protease Assay Kit II (Pierce). For inhibitory assays, the gliotoxin stock solution was prepared in dimethyl sulfoxide. Gliotoxin at different concentrations was preincubated with 5 μg of the indicated peptide-amc molecules for 15 min at room temperature in 0.1 M Hepes pH 7.8. The respective substrates were added to give a final volume of 200 μl, and activities were measured using fluorogenic peptides as described above.

In vitro *ubiquitin transfer reactions*

Recombinant yeast E1 (0.1 μg) and CDC34 (2 μg) were incubated with ^{32}P-radiolabeled GST–ubiquitin (3500 cpm per 20 μl reaction volume) expressed from pGex2TK vector (Pharmacia) in 20 mM Tris pH 7.5, 2 mM ATP, and 10 mM $MgCl_2$. Reaction mixtures were incubated at

37°C for 30 min and fractionated using SDS–PAGE. Gels were dried and analysed by autoradiography.

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