Production of the Invasive Aspergillosis Biomarker Bis(methylthio)gliotoxin Within the Genus Aspergillus: In Vitro and in Vivo Metabolite Quantification and Genomic Analysis

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Gliotoxin (GT) is a fungal secondary metabolite that has attracted great interest due to its high biological activity since it was discovered by the 1930s. An inactive derivative of this molecule, bis(methylthio)gliotoxin (bmGT), has been proposed as an invasive aspergillosis (IA) biomarker. Nevertheless, studies regarding bmGT production among common opportunistic fungi, including the Aspergillus genus, are scarce and sometimes discordant. As previously reported, bmGT is produced from GT by a methyl-transferase, named as GtmA, as a negative feedback regulatory system of GT production. In order to analyze the potential of bmGT detection to enable identification of infections caused by different members of the Aspergillus genus we have assessed bmGT production within the genus Aspergillus, including A. fumigatus, A. niger, A. nidulans, and A. flavus, and its correlation with gtmA presence. In order to validate the relevance of our in vitro findings, we compared bmGT during in vitro culture with the presence of bmGT in sera of patients from whom the Aspergillus spp. were isolated. Our results indicate that most A. fumigatus isolates produce GT and bmGT both in vitro and in vivo. In contrast, A. niger and A. nidulans were not able to produce GT or bmGT, although A. niger produced bmGT from an exogenous GT source. The frequency and amount of bmGT production in A. terreus and A. flavus isolates in vitro was lower than in A. fumigatus. Our results suggest that this defect could be related to the in vitro culture conditions, since isolates that did not produce bmGT in vitro were able to synthetize it in vivo. In summary, our study indicates that bmGT could be very useful to specifically detect the presence of A. fumigatus, the most prevalent agent causing IA. Concerning A. terreus and A. flavus a higher number of analyses from sera from infected patients will be required to reach a useful conclusion.

Keywords: bis(methylthio)gliotoxin, Aspergillus spp., gtmA, invasive aspergillosis, biomarker
INTRODUCTION

More than 20 years ago the first invasive aspergillosis (IA) biomarker, galactomannan (GM), was developed based on an enzyme linked immunosorbent assay (Stynen et al., 1995). It stirred up the diagnosis of this lethal infectious disease, as it allowed to detect the infection when combined with clinical signs and symptoms (Maertens et al., 1999, 2002). During the last few years, the biomarker weaponry has arisen with the signs and symptoms (Maertens et al., 1999, 2002). During the allowed to detect the infection when combined with clinical

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the last one, it is commonly used in *Aspergillus* cultures *in vitro*. Thus, we decided to compare both in order to discard effects relative to specific culture media conditions *in vitro*.

**Bis(methylthio)gliotoxin Production From Exogenous Gliotoxin**

Bis(methylthio)gliotoxin production from an exogenous source of GT was assessed in a total of 35 isolates of the species complexes *A. flavus* (*n* = 12), *A. terreus* (*n* = 9), *A. niger* (*n* = 8), and *A. nidulans* (*n* = 6). Conidial inoculum was prepared as described above and added to 50 mL culture flasks with Czapek Dox Broth (+ glutamine + HEPES). These cultures were incubated at 37°C for 45 h. At 45 h, GT was added to a final concentration of 2.5 mg/L and methanol was added as solvent control. At 0, 3, and 6 h, 2 mL aliquots of supernatant were taken and frozen until GT and bmGT analysis.

**Detection of Bis(methylthio)gliotoxin in Sera From IA Patients**

We assessed sera from patients hospitalized in the Miguel Servet University Hospital with probable/proven IA according to the EORTC/MSG definitions (De Pauw et al., 2008). We included in the study those cases with *Aspergillus* spp. growth in clinical samples. Serum were prospectively collected and frozen at −20°C until GT and bmGT detection. All protocols were supervised and approved by the Ethics Committee of Clinical Research from Aragón (CEICA), number PI15/0203.

**Metabolite Identification and Quantification by High Performance Thin Layer Chromatography (HPTLC)**

Gliotoxin and bis(methylthio)gliotoxin detection and quantification were performed both in serum and supernatant samples by HPTLC as described by Domingo et al. (2012). Briefly, GT and bmGT were extracted together using dichloromethane. After agitation and two phase's separation, non-aqueous phase was added onto silica gel plates. Then, they were developed with an ultraviolet scanning densitometry (TLC Scanner 3, Camag; λ = 280 and 367 nm; linear scanning). GT and bmGT identification was performed by retention time and spectral analysis and quantification was performed by peak area under curve analysis using Camag's personal computer software.

**Genetic Detection of *gtmA* Gene**

Chromosomal DNA of *Aspergillus* spp. isolates was extracted using cetyltrimethylammonium bromide (Sigma-Aldrich, St. Louis, MO, United States). The specie-specific primers used for *gtmA* detection are summarized in Table 1, along with expected amplicon length. PCR was performed using HotTaq Master Mix, (IBIAN Technologies, Zaragoza, Aragón, Spain). An initial denaturation of 2 min at 94°C was followed by 35 cycles at 94°C for 30 s, 56°C for 15 s, and 72°C for 1 min. DNA amplification products were visualized after electrophoresis on 2% agarose gels.

| Name            | Sequence (5′–3′)                  | Amplification length (bp) |
|-----------------|----------------------------------|---------------------------|
| *A. terreus* Fw | TCG GAG GCC GTA AAC CG            | 291                       |
| *A. terreus* Rv | GTG TCA GCC CTG CAT AC           |                           |
| *A. flavus* Fw  | TGC TCA GGG AAG AGA TTA AAA GC   | 289                       |
| *A. flavus* Rv  | TCG TCA GGG AAG AGA TTA AAA GC   | 289                       |
| *A. niger* Fw   | TGC TCA GGG AAG AGA TTA AAA GC   | 225                       |
| *A. niger* Rv   | TGC TCA GGG AAG AGA TTA AAA GC   | 225                       |
| *A. fumigatus* Fw | GCC TCT GAA AGA TCT GAA AG      | 293                       |
| *A. fumigatus* Rv | GCC TCT GAA AGA TCT GAA AG      | 293                       |
| ITS 1 Fw        | TCG GTA GGT GAA CCT GCC G        | 565 to 613                |
| ITS 4 Rv        | TCC TCC GCT TAT TGO TAT G        |                           |

UView 6x loading dye (Bio-Rad, Hercules, CA, United States) was used for nucleic acid staining. As a length standard 0.1–1 kbp molecular mass marker was used.

**RESULTS**

Gliotoxin and Bis(methylthio)gliotoxin Production by *Aspergillus* spp. Isolates

The production of GT and bmGT was tested in culture supernatants of 252 *Aspergillus* isolates from the species complexes *A. fumigatus, A. flavus, A. terreus, A. niger,* and *A. nidulans* after 4 days of incubation. Non-cryptic *A. fumigatus* isolates (*n* = 101) produced GT and bmGT at highest frequencies, 77.23% and 84.16%, respectively. Among the five cryptic species analyzed: *A. calidoustus* (*n* = 1), *A. fumigatiaffinis* (*n* = 3), *A. lentulus* (*n* = 11), *N. udagawae* (*n* = 2), and *A. novofumigatus* (*n* = 1), all but the last produced GT and/or bmGT. *A. fumigatiaffinis* seemed to be the most frequent GT and bmGT producing species, as the three tested isolates (100%) produced bmGT. In contrast, just two of the eleven isolates (18%) of *A. lentulus* produced bmGT. BmGT was also more frequently detectable than GT in culture supernatants of *A. flavus*, 11.11% vs. 8.33%, respectively. In contrast, *A. terreus* produced GT more frequently than bmGT (22.86% vs. 2.86% respectively) (Figure 1). Notably, none of the *A. niger* and *A. nidulans* isolates tested produced GT and/or bmGT. All cultures were analyzed by HPTLC, a method to detect GT and bmGT previously optimized and validated versus LC-MS (Domingo et al., 2012), and some of the results in selected culture isolates of *A. fumigatus, A. niger, A. nidulans,* and *A. flavus,* were confirmed by LC-MS (data not shown). The absence of GT and/or bmGT production was not specific for the culture conditions *in vitro* since culture
of GT/bmGT negative fungal isolates employing Czapek Dox Broth yielded similar results (data not shown). In addition, the differences were not due to different fungal growth since cell cultures showed a similar behavior and growth as analyzed by XTT reduction assay. Optimal culture conditions, as well as analytical specificity of the method, were confirmed by employing cell cultures from an A. fumigatus gliP deletion mutant, which is unable to produce GT and bmGT (Sugui et al., 2007).

A. fumigatus isolates also produced GT and bmGT in higher concentration than other Aspergillus spp. did. These differences were statistically significant \( p < 0.05 \) (Figure 2). The mean concentration of GT was 2.26 ± 0.40 mg/L and of bmGT was 3.45 ± 0.44 mg/L for A. fumigatus. A. flavus isolates yielded a mean concentration of 0.14 ± 0.13 mg/L of GT and 0.39 ± 0.31 mg/L of bmGT. The mean concentration of GT and bmGT were 0.79 ± 0.34 mg/L and 0.07 ± 0.07 for A. terreus.

### Bis(methylthio)gliotoxin Production From Exogenous Gliotoxin

In order to confirm the results obtained in cell cultures concerning bmGT production, we analyzed the ability of some fungal isolates to generate bmGT from an external GT source as well as the presence of methyltransferase genes. This is of special utility to find out whether the Aspergillus spp. that did not produce GT and bmGT \( (A. \ niger \ and \ A. \ nidulans) \) are also unable to methylate exogenous GT. This finding would mean that these isolates do not express methyl-transferase activity and, thus, confirm that they are unable to generate bmGT. Moreover, this would indirectly suggest that they are also unable to generate GT, since GT methylation has been proposed as a negative feedback regulatory system, inherent to all GT-producing species.

First, we analyzed if fungal isolates presented GT methyl-transferase activity by adding pure GT and monitoring the generation of bmGT. The ability to produce bmGT from an exogenous source of GT was assessed in the isolates of A. flavus, A. terreus, A. niger, and A. nidulans that did not produce GT or bmGT in the previous experiment. All isolates from the A. flavus \( (n = 12) \) and A. terreus species \( (n = 9) \) were able to methylate exogenous GT in order to produce bmGT. Among A. niger isolates, this ability was less consistent, nevertheless, 5/8 isolates (62.5%) showed such ability. Finally, none of the A. nidulans isolates \( (n = 6) \) methylated GT to generate bmGT (Figure 3). This result confirms that A. nidulans does not express methyl-transferase activity and, thus, is unable to methylate exogenous GT in order to produce bmGT. Among A. niger isolates, this ability was less consistent, nevertheless, 5/8 isolates (62.5%) showed such ability. Finally, none of the A. nidulans isolates \( (n = 6) \) methylated GT to generate bmGT (Figure 3). This result confirms that A. nidulans does not express methyl-transferase activity and, thus, is unable to methylate exogenous GT in order to produce bmGT. Among A. niger isolates, this ability was less consistent, nevertheless, 5/8 isolates (62.5%) showed such ability. Finally, none of the A. nidulans isolates \( (n = 6) \) methylated GT to generate bmGT (Figure 3). This result confirms that A. nidulans does not express methyl-transferase activity and, thus, is unable to methylate exogenous GT in order to produce bmGT. Among A. niger isolates, this ability was less consistent, nevertheless, 5/8 isolates (62.5%) showed such ability. Finally, none of the A. nidulans isolates \( (n = 6) \) methylated GT to generate bmGT (Figure 3). This result confirms that A. nidulans does not express methyl-transferase activity and, thus, is unable to methylate exogenous GT in order to produce bmGT.
Among bmGT producing isolates, immediately after GT addition \((t = 0 \text{ h})\), this was recovered in a mean concentration of \(0.86 \pm 0.04 \text{ mg/L}\). None of the isolates produced bmGT at this time (Figure 4). At 3 h after GT addition, GT concentration decreased and bmGT concentration increased. This observation continued at 6 h, when the maximum bmGT and the minimum GT concentrations were detected. There were no differences between mean concentration of GT and bmGT among species \((p > 0.05)\) indicating a similar methylating activity.

**Comparison of Serum bmGT Detection in Vivo With bmGT Production in in Vitro Cultures**

Our results confirm that most *A. fumigatus* isolates and some *A. terreus* and *A. flavus* isolates were able to endogenously and exogenously produce bmGT. However, the frequency of bmGT production within *A. terreus* and *A. flavus* isolates was much less than in *A. fumigatus* isolates. Since the analyses of the genes involved in GT synthesis is difficult due to the complexity of the pathways involved, we decided to analyze if the isolates that did not produce endogenously GT and bmGT in *vitro*, were able to synthesis bmGT in humans *in vivo*. To this aim, we included six cases of probable/proven IA with mycological growth from whom *in vitro* cultures had been established and analyzed. Serum bmGT concentration for these patients, fungal isolation, sample type and fungal ability to produce bmGT *in vitro* (de novo and from exogenous GT) as well as detection of *gtmA* gene and the MT-ii homolog are summarized in Table 2.

All, but one serum, were positive for bmGT. This serum belonged to a patient with probable IA diagnosed by *A. fumigatus* growth in bronchial aspirate. The other three patients with *A. fumigatus* isolation had positive bmGT (range 0.19–13.68 mg/L). There was a case of IA by *A. flavus* and a case of IA by *A. terreus*. Both had detectable bmGT in serum. Notably, these isolates corresponded to those ones in which we were not able to detect either endogenous GT or bmGT during *in vitro*
culture. Nevertheless, all of them methylated the exogenous GT and carried the mt-II methyl-transferase gene, as seen in Figure 5. Of note, those isolates which produced bmGT in higher amounts in vitro, did not correlate with the highest bmGT production in vivo. All the clinical isolates from A. fumigatus showed the ability to methylate exogenous GT and carry the gtmA gene.

Finally, in order to confirm that A. nidulans and A. niger did not produce GT and, thus, are unable to endogenously synthesize bmGT, we analyze the presence of gliP gene (a critical gene within the gli cluster responsible for GT synthesis) by PCR. None of the isolates from A. niger and A. nidulans carry the gliP gene (data not shown) confirming that they are unable to produce GT and bmGT, as found in the cell culture analysis (Figure 1). Moreover, a bioinformatic analyses searching for the presence of gli cluster homology sequences in the genome of sequenced A. niger and A. nidulans strains, yielded negative results, confirming our experimental data and in line with previous findings (De Pauw et al., 2008; Manzanares-Miralles et al., 2016). In contrast, sequences with some homology to gli cluster were found in both A. terreus and A. flavus genomes (data not shown) as previously indicated (Patron et al., 2007).

### DISCUSSION

Despite recent advances, the lack of a single gold standard technique and the limitations of the available ones, make diagnosis of IA still challenging (Maertens et al., 2016; Mercier and Maertens, 2017). In recent years, new metabolite based diagnostic tools have been under research, such as GT or volatile organic compounds (Lewis et al., 2005a; Chambers et al., 2009).

Regarding GT, its high biological reactivity and its potential ability to interact with cells and tissues (Domingo et al., 2012) make it hard to detect in body fluids (Scharf et al., 2012). This limitation is overcame by bmGT, which is more stable and reliably detected in serum (Domingo et al., 2012). It is known that A. fumigatus produces GT in the highest concentrations and more frequently than other Aspergillus species (Lewis et al., 2005b; Kupfahl et al., 2008). This conclusion has been supported by our results, in which 77% of the A. fumigatus isolates were GT-producers in significantly higher concentrations and frequencies than those obtained for A. flavus and A. terreus species complexes, the other Aspergillus-producing GT species. With reference to bmGT, A. fumigatus was also the most common bmGT producing complex. Notably, and in line with previous findings (De Pauw et al., 2008; Manzanares-Miralles et al., 2016), neither A. niger or A. nidulans strains were able to endogenously produce GT and/or bmGT, although methyl-transferase activity was found in A. niger isolates when using exogenous GT. These results are supported by both PCR analyses and bioinformatic studies confirming the absence of the gli cluster in these species. These findings contrast to previous studies in which a high proportion of A. niger isolates were shown to produce GT (Kupfahl et al., 2008). We have no explanation for these contradictory findings although, in line with our findings, Kupfahl et al. (2008) did not detect gliP gene in A. niger, which has been shown to be critical for GT synthesis, at least in A. fumigatus isolates (Sugui et al., 2007).

Some authors have been interested in the secondary metabolite profiles of cryptic species of the Fumigati section. Unlike other authors, we found out that A. lentulus and A. fumigattiaffinis were able to produce GT and bmGT (Larsen et al., 2007; Sugui et al., 2010; Tamiya et al., 2015). Regrettably, we...
just analyzed one isolate of A. calidoustus and A. novofumigatus. The A. calidoustus isolate was GT and bmGT producer, but A. novofumigatus was not. This result does not rule out the ability to produce GT and bmGT by A. novofumigatus since culture conditions (medium, aeration, temperature, sampling time ...) affect to secondary metabolite synthesis (Belkacemi et al., 1999; Watanabe et al., 2004). This could explain the low GT and bmGT detection among non-A. fumigatus species since all experiments were performed in the same conditions and confirmed employing other culture protocols.

Aiming to avoid such a limitation, we analyzed the ability to produce bmGT from an exogenous source of GT among non-toxigenic isolates. We detected bmGT in culture filtrates of all the A. flavus and A. terreus analyzed, thus suggesting a consistent ability to produce bmGT. To our knowledge no specific methyltransferases had been described to date for these species. Nevertheless, it has been described an ortholog and a homolog of GtmA for A. terreus and A. flavus by bioinformatics analysis but it is the first time in which its expression has been described. In our study, A. niger also showed the ability to methylate GT, but less frequently. Curiously, none of the A. nidulans isolates analyzed produced bmGT even when it has been described that a specific methyltransferase (and the encoding gene) able to produce bmGT from GT for this species (Manzanares-Miralles et al., 2016). This discrepancy could be due to the known fact that culture conditions do not reflect the genetic potential and that not all the strains of the same species have the same metabolic profile (Bergmann et al., 2007). Indeed, secondary metabolism confers a survival benefit to the producing isolate and the in vitro culture conditions are not optimal to activate this survival pathway, depending on the Aspergillus spp. and/or isolate.

In order to overcome the limitations of the in vitro culture to analyze secondary metabolism, we have employed some clinical isolates from patients with probable/proven IA and compared bmGT production in in vitro culture with bmGT in serum from those patients. In this scenario, where fungi has to colonize the host and adapt itself to the new environmental conditions, the fungi would activate secondary metabolism and display all potential virulence factors such a GT (Cramer et al., 2006; Sugui et al., 2007) In these conditions, all but one of the seven probable/proven patients had bmGT detectable in serum, even those that did not produce GT and bmGT in vitro. Importantly, all of them were able to produce bmGT from exogenous GT and carried an ortholog of GtmA, MT-ii.

In summary, our findings indicate that bmGT production is useful to diagnose IA caused by A. fumigatus and, at some extent, by A. terreus and A. flavus, although at a much lower frequency, since they present the ability to methylate GT and endogenously produce bmGT in vitro and in vivo. Moreover, and pending of validation with a higher number of samples, our novel findings indicate that conclusions about the expression of molecules that could be used as potential diagnostic biomarkers based on in vitro fungal cultures cannot be reached unless they are confirmed in proper in vivo studies employing animal models or patients suffering from IA.

AUTHOR CONTRIBUTIONS

MV-G and SR carried out the experiments. PM helped with genomic analysis and CC helped with in vitro experiments. MD performed the HPTLC analysis. MV-G wrote the manuscript with support from AR, JP, and EG. JM provided and characterized different isolates of Aspergillus spp. JP and EG conceived the original idea and supervised the project with the support of AR.

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**Conflict of Interest Statement:** MD, JP, and EG are co-inventors of a patent licensed to Blackhills Diagnostic Resources S.L. that protects the use of bmGT to diagnose IA (PCT/EP2012/058,247).

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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