Electronic Supplementary Material (ESI) for Metallomics.

Running Head: Zinc chelation by dithiol gliotoxin.

Systems Impact of Zinc Chelation by the Epipolythiodioxopiperazine Dithiol Gliotoxin in *Aspergillus fumigatus*: A New Direction in Natural Product Functionality.

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KEYWORDS holomycin, metalloenzyme, NRPS, antimicrobial resistance, AMR, quantitative proteomics.

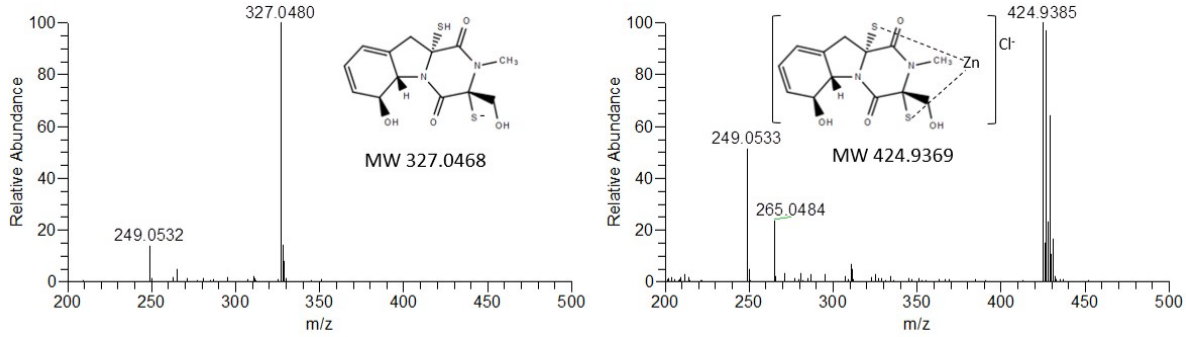


Figure S1. High mass accuracy spectra of gliotoxin (left) and DTG complexed with Zn^{2+} (Cl^- adduct) (right). Calculated accurate mass indicated under the structure of each molecule.

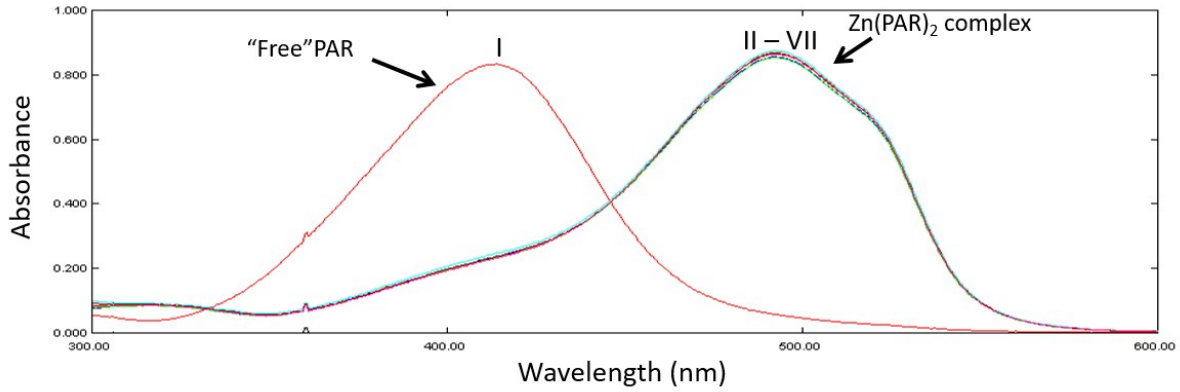
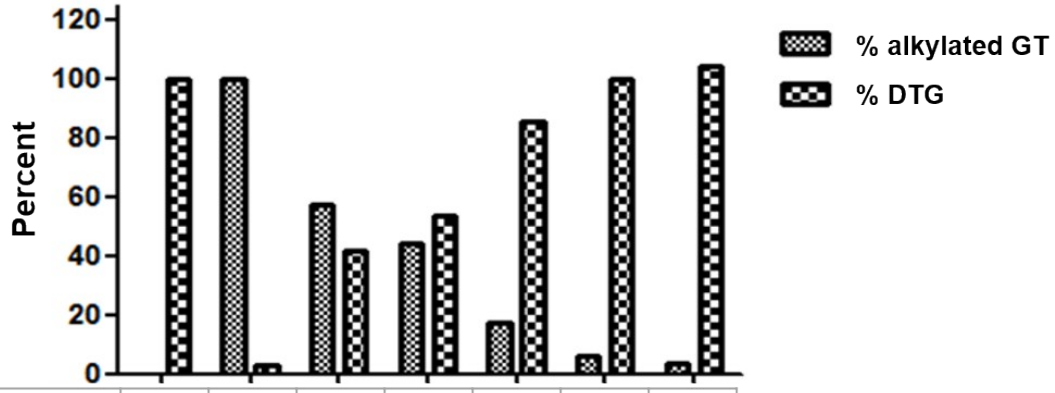


Figure S2. Overlaid UV-vis profiles of PAR alone (I; red), and $Zn(PAR)_2$ (II). Oxidised gliotoxin added in increased concentration to $Zn(PAR)_2$ (1-5 molar equivalents of oxidised gliotoxin to Zn^{2+}; III-VII, respectively). Oxidised gliotoxin causes no displacement of Zn^{2+} from the $Zn(PAR)_2$ complex.



DTG (mM)	0.3	0.3	0.3	0.3	0.3	0.3	0.3
IAA (mM)	0	3	3	3	3	3	3
Zn^{2+} (mM)	0	0	0.1	0.15	0.3	0.6	0.9
Ratio Zn^{2+}:DTG			0.33:1	0.5:1	1:1	2:1	3:1

Figure S3. Zn^{2+} inhibition of iodoacetamide (IAA)-mediated alkylation of dithiol gliotoxin (DTG) shown in Figure 2D. IAA converts DTG to alkylated GT, however increasing free Zn^{2+} (0.33 to 3 molar excess) inhibits this alkylation reaction, as determined by the increased amount of DTG detected with increasing Zn^{2+} concentration.

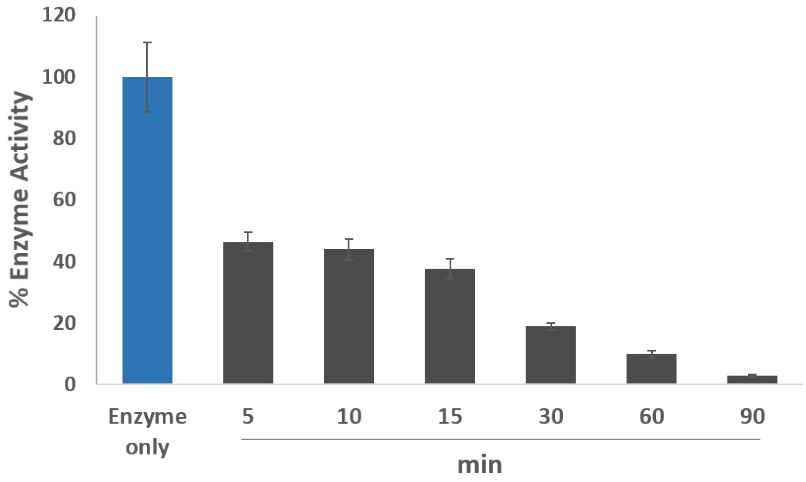


Figure S4. Time dependent inhibition of alkaline phosphatase by dithiol gliotoxin. Alkaline phosphatase was incubated in the absence (blue) and presence (black) of dithiol gliotoxin (DTG; 50 µM) up to 90 min prior to assay.

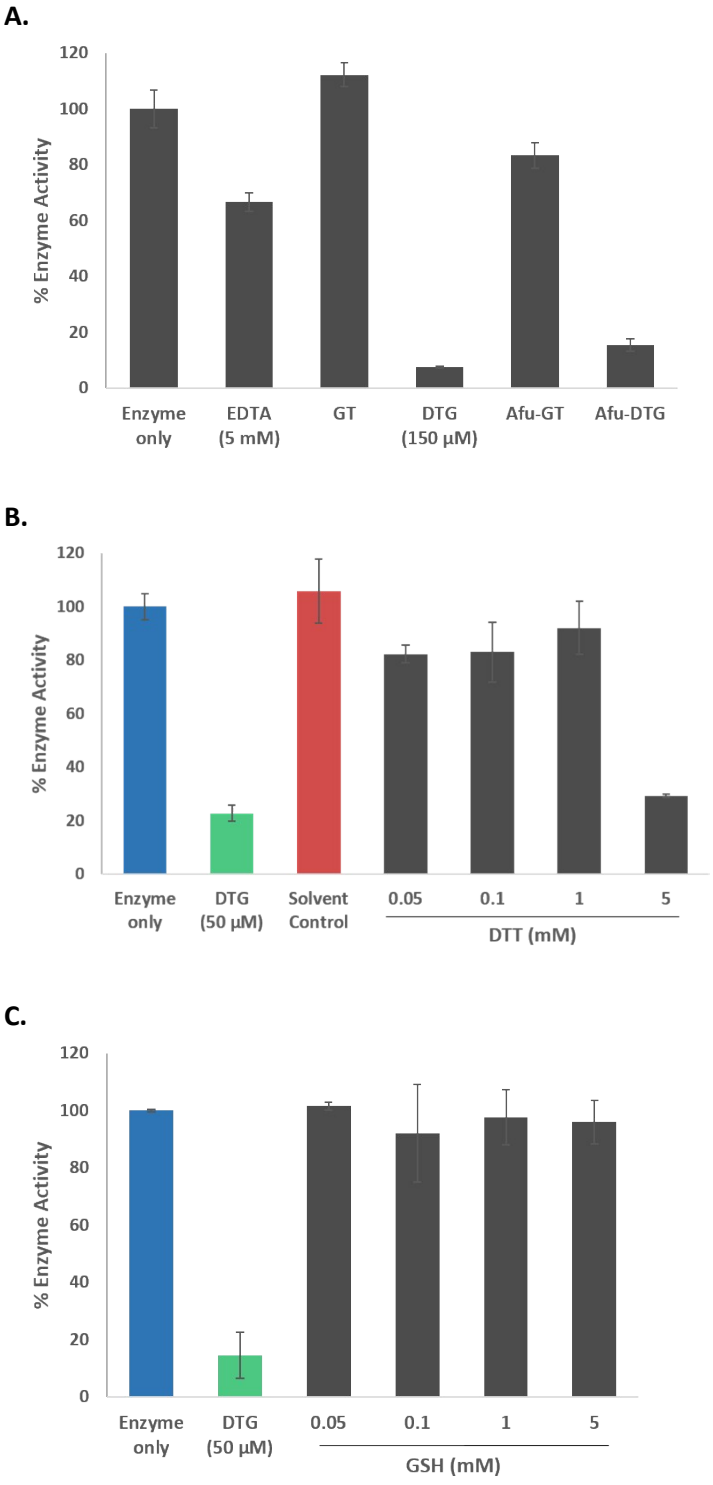


Figure S5. Effect of chelating agents and reducing agents on alkaline phosphatase (AP) activity. **A.** EDTA (5 mM) partially inhibits alkaline phosphatase activity, while gliotoxin (GT) induces no enzyme inhibition. TCEP-reduced gliotoxin (DTG; 150 µM) substantially inhibits AP activity. Similarly gliotoxin derived from *A. fumigatus* culture supernatants (Afu-GT) showed an increase in enzyme inhibition following TCEP-mediated reduction (Afu-DTG). Impact of different concentrations of dithiothreitol (DTT; **B**) or glutathione (GSH; **C**) (0.05, 0.1, 1.0 and 5.0 mM) on the activity of alkaline phosphatase. Solvent controls contain equivalent concentrations of methanol and TCEP as the DTG sample.

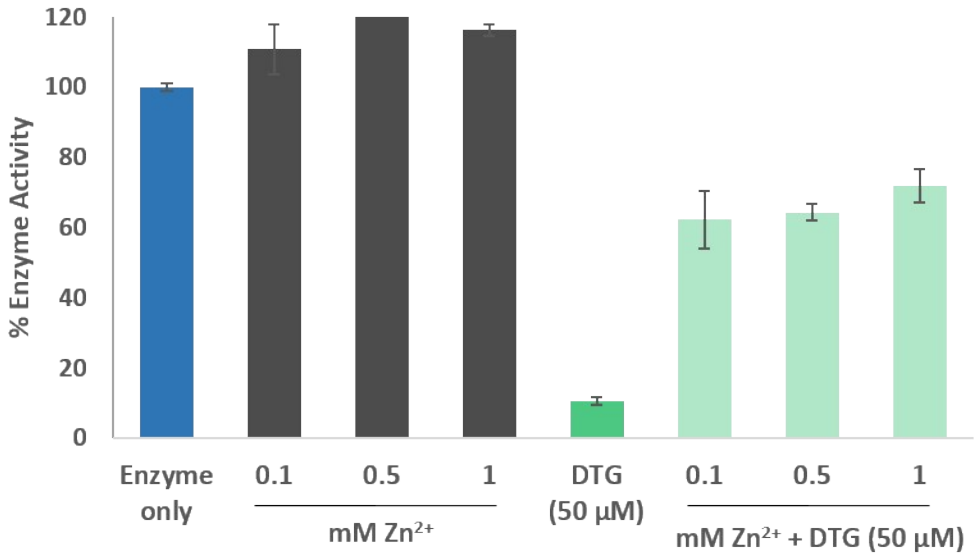


Figure S6. Zn^{2+} partially recovers AP enzyme activity following DTG-induced inhibition. Pre-incubation of AP with DTG (15 minutes) causes substantial inhibition of enzyme activity (dark green). Subsequent addition of Zn^{2+} (0.1-1 mM) partially recovers this activity (light green). This complements the result shown in Figure 3C, which demonstrates that pre-incubation of DTG with Zn^{2+} completely protects AP from DTG-associated inhibition.

A.

B.

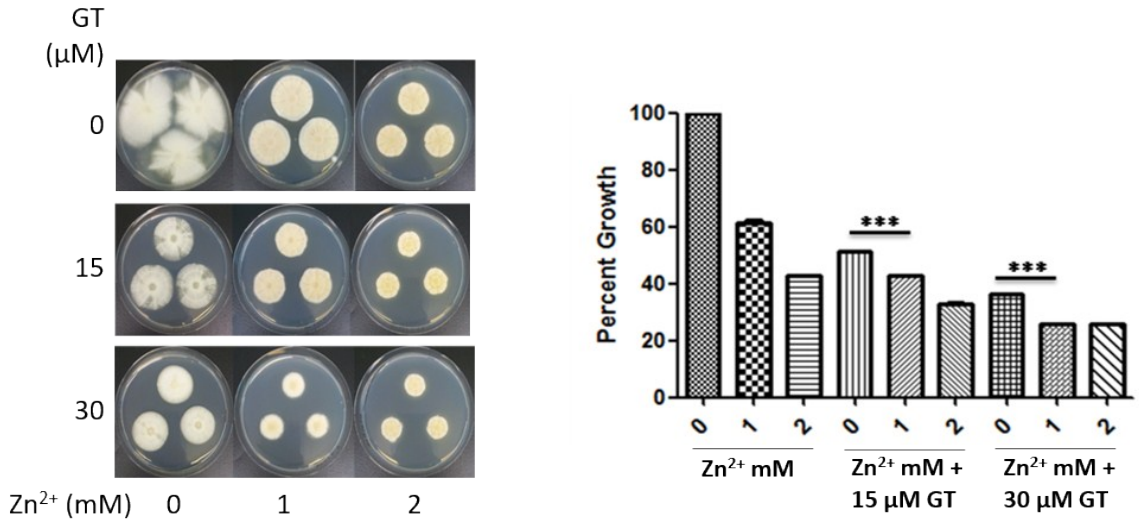


Figure S7. Gliotoxin (15 and 30 µM) acts synergistically with Zn^{2+} to significantly ($p < 0.005$) inhibit growth of *A. fumigatus* Δ*gliT*. **A.** Plate assays showing the effect of Zn^{2+} alone on *A. fumigatus* Δ*gliT growth* at 96 h, and the combinatorial effect with gliotoxin. **B.** Graphical representation of same in terms of relative growth percentages.

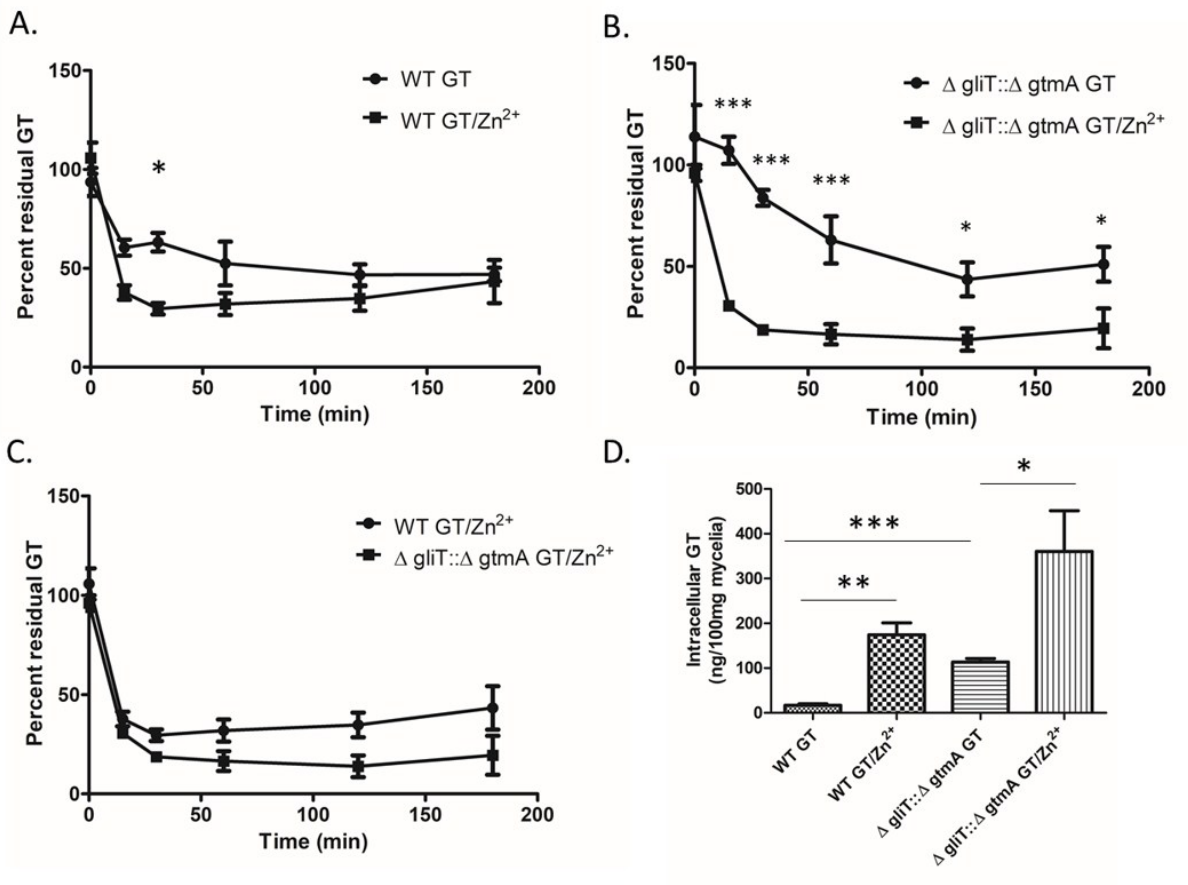
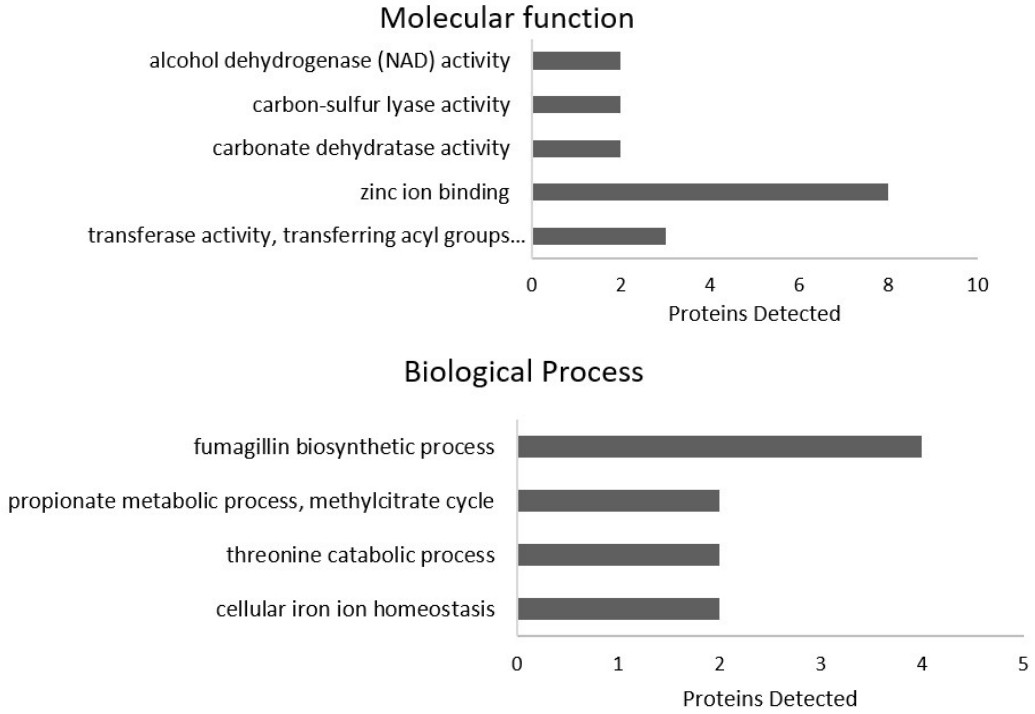


Figure S8. Zn^{2+} affects gliotoxin uptake and secretion in *A. fumigatus*. **A.** Zn^{2+} increases the rate and extent of gliotoxin uptake by *A. fumigatus* wild-type at 30 min. **B.** Zn^{2+} increases the rate and extent of gliotoxin uptake by *A. fumigatus* Δ*gliT*::Δ*gtmA*. **C.** Comparative effect of Zn^{2+} on gliotoxin uptake and efflux by *A. fumigatus* wild-type and Δ*gliT*::Δ*gtmA*. **D.** Relative accumulation of gliotoxin in fungal mycelia following exposure to exogenous gliotoxin (+/- Zn^{2+}). Zn^{2+} addition results in significantly greater accumulation of gliotoxin in both *A. fumigatus* wild-type and Δ*gliT*::Δ*gtmA*. The impaired efflux observed (S8C) for *A. fumigatus* Δ*gliT*::Δ*gtmA*, compared to wild-type, is accompanied by >2x intracellular accumulation of gliotoxin. Significance was determined by 2-way ANOVA and Bonferroni post-tests * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

A.



B.

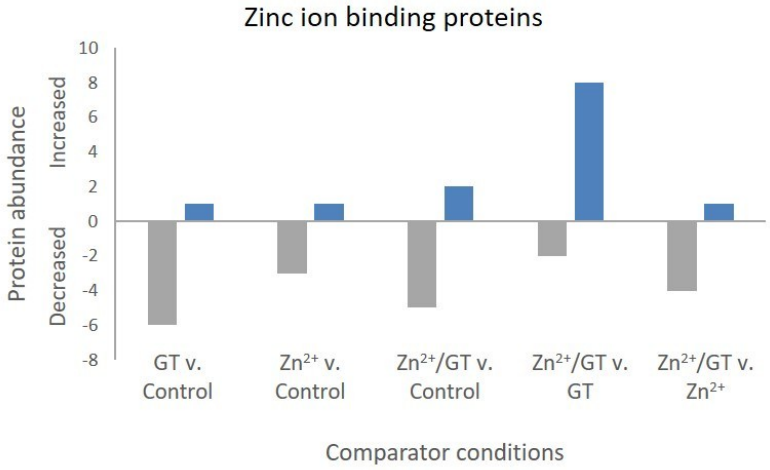
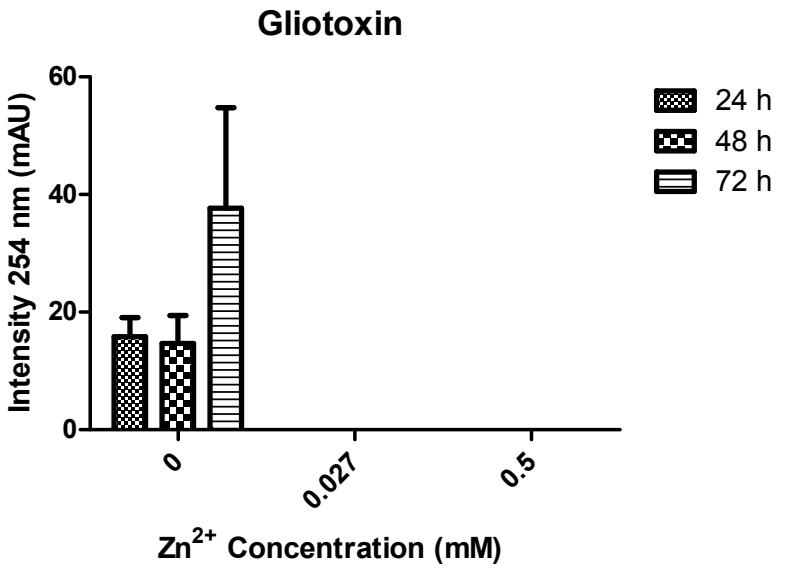


Figure S9: Functional analysis (FungiFun2: GO annontation) of proteins with altered abundance following combinatorial treatment (Zn^{2+}/GT) of *A. fumigatus* Δ*gliT* compared to gliotoxin alone. Qualitative and quantitative results are combined and analysis was performed on proteins showing differential abundance. **A.** Significantly enriched functional groups among proteins with higher abundance in Zn^{2+}/GT treatment compared to GT alone. Enriched categories ($p < 0.05$) with more than 1 protein/category shown in order of ascending p value. **B.** Proteins with zinc-binding activity (GO term: 0008270) with increased (above x-axis) or decreased (below x–axis) abundance in *A. fumigatus* Δ*gliT* in each of the comparator conditions. Qualitative and quantitative results are combined to generate proteins with increased or decreased abundance. Zinc-binding proteins (n=8) were significantly increased in abundance in the combinatorial condition (Zn^{2+}/GT) compared to gliotoxin treatment alone ($p = 0.005$).

A.



B.

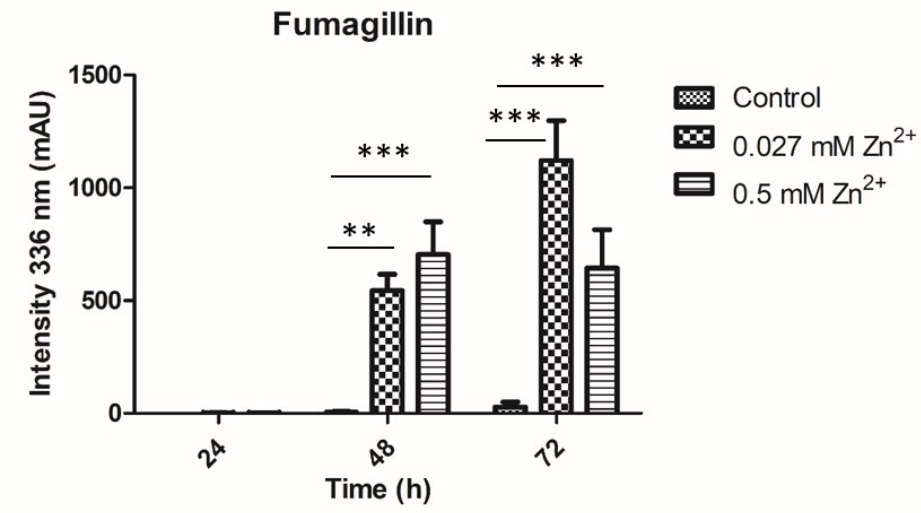


Figure S10. A. Relative gliotoxin levels in organic extracts obtained from *A. fumigatus* cultures (grown ± 0.027 mM or 0.5 mM $ZnSO_4$) in Czapek-Dox media (24, 48, 72 h). Detection wavelength: 254 nm. **B.** Relative fumagillin levels in organic extracts obtained from *A. fumigatus* cultures (grown ± 0.027 mM or 0.5 mM $ZnSO_4$) in Czapek-Dox media (24, 48, 72 h). Detection wavelength: 336 nm. A 2-way ANOVA with Bonferroni post-tests was used to compare results. ** $p < 0.01$, *** $p < 0.001$