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1 **A novel polyaminocarboxylate compound to treat murine pulmonary aspergillosis by**  
2 **interfering with zinc metabolism**

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20

21 **ABSTRACT**

22 *Aspergillus fumigatus* can cause pulmonary aspergillosis in immunocompromised patients  
23 and is associated with a high mortality rate due to the lack of reliable treatment options. This  
24 opportunistic pathogen requires zinc in order to grow and cause disease. Novel compounds  
25 that interfere with fungal zinc metabolism may therefore be of therapeutic interest. We  
26 screened chemical libraries containing 59223 small molecules using a resazurin assay that  
27 compared their effects on an *A. fumigatus* wild type strain grown under zinc-limiting  
28 conditions and on a zinc transporter knockout strain grown under zinc-replete conditions to  
29 identify compounds affecting zinc metabolism. After a first screen 116 molecules were  
30 selected whose inhibitory effect on fungal growth was further tested by using luminescence  
31 assays and hyphal length measurements to confirm their activity, as well as to toxicity assays  
32 on HeLa cells and mice. Six compounds were selected following a re-screening, two of which  
33 were pyrazolones, two were porphyrins and two were polyaminocarboxylates. All three  
34 groups showed good *in vitro* activity but only one of the polyaminocarboxylates was able to  
35 significantly improve the survival of immunosuppressed mice suffering from pulmonary  
36 aspergillosis. This two-tier screening approach led us to the identification of a novel small  
37 molecule with *in vivo* fungicidal effects and low murine toxicity that may lead to the  
38 development of new treatment options for fungal infections either by administration of this  
39 compound as a monotherapy or as part of a combination therapy.

40

41 **INTRODUCTION**

42 *Aspergillus fumigatus* is a ubiquitous, opportunistic fungal pathogen. It can cause invasive  
43 aspergillosis in immunocompromised individuals and is responsible for over 200,000 life-

44 threatening infections per year (9). The preferred drug for treating this infection is  
45 voriconazole, which inhibits ergosterol synthesis, though amphotericin B, which binds to  
46 ergosterol, and echinocandins, which inhibit glucan synthesis, are alternatives (11).  
47 However, all these treatment options have limitations. Azole resistance is emerging across  
48 the world which would negatively impact voriconazole-based treatments, amphotericin B is  
49 associated with significant toxicity, and echinocandins are only able to arrest growth of the  
50 pathogen (4). Novel treatment options are thus urgently needed in order to combat invasive  
51 aspergillosis.

52 Zinc is the second most abundant transition metal after iron in humans and is essential for  
53 all organisms as it is required for enzymes of all functional classes (16). Free zinc is tightly  
54 regulated within the human body and is only found at a concentration of 10 picomoles in  
55 order to prevent pathogens from acquiring it (45), a process termed nutritional immunity  
56 (22). In addition, infiltrated neutrophils in fungal abscesses release high amounts of  
57 calprotectin, a peptide heterodimeric protein that binds zinc and manganese with an  
58 extremely high affinity and limits their availability to pathogens (16). In order to obtain zinc,  
59 *A. fumigatus* utilizes three plasma membrane zinc transporters encoded by the *zrfA*, *zrfB*,  
60 and *zrfC* genes (3), which are regulated by the ZafA transcriptional activator (32). Loss of *zrfC*  
61 results in a partial loss of virulence, whereas the deletion of three genes results in a  
62 complete loss of virulence, which shows that they all function together to obtain zinc from  
63 the host (3).

64 Calprotectin, which has a zinc chelating activity, has been used to inhibit the growth of *A.*  
65 *fumigatus* in the corneas of immunocompetent mice (14). In addition, our group has used  
66 the zinc chelators N,N,N',N'-tetrakis(2-pyridinylmethyl)-1,2-ethanediamine (TPEN) and

67 phenanthroline to successfully treat invasive pulmonary aspergillosis in mouse models (26).  
68 These findings support the suggestion that a reduction in the availability of zinc could have  
69 clinical applications for the treatment of aspergillosis (42). Following these promising  
70 findings, we carried out the screening of small molecule libraries in order to find additional  
71 compounds that targeted the *A. fumigatus* zinc metabolism. We first performed a resazurin  
72 assay as it is recommended for *A. fumigatus* screens (40), and has been successfully used to  
73 test the effects of antifungal drugs on *A. fumigatus* strains (15, 46), as well as to screen  
74 chemical libraries for novel antifungals (31, 38). This was followed by more exhaustive *in*  
75 *vitro* experiments utilizing luciferase and hyphal length measurements and then by *in vivo*  
76 tests on mouse models (26).

77

## 78 RESULTS

79 **Screen and subsequent assays revealed six compounds of interest.** The primary resazurin  
80 screen comprised 59,223 compounds from commercial libraries (Chem-X-Infinity and  
81 Prestwick) and from the French academic library “Chimiothèque Nationale” (21). At an  
82 average concentration of  $7.0 \pm 3.5 \mu\text{M}$ , only 116 compounds were found to inhibit the growth  
83 of *A. fumigatus* wild type (AF14<sup>LUC</sup>) with no added zinc, but permit the growth of the *A.*  
84 *fumigatus* triple zinc transporter knockout (AF721<sup>LUC</sup>) with 100  $\mu\text{M}$  of added zinc. 91% of  
85 these compounds were validated using resazurin gradient concentration assays. These were  
86 followed up by luciferin gradient assays, where 15 compounds demonstrated at least a  
87 twofold difference in  $\text{MIC}_{50}$  between the wild type and triple knockout. Out of 15  
88 compounds, 8 compounds were effective using hyphal length measurements. However, one  
89 compound was rejected due to high cytotoxicity on HeLa cells, another due to high toxicity

90 on mice and a third was omitted due to the very limited availability. This left 6 compounds  
91 that were not toxic to HeLa cells (Fig. S1) and that were used in further experiments. These  
92 compounds belonged to three different chemical series: two pyrazolones, two porphyrins  
93 and two polyaminocarboxylates.

94 **Pyrazolone family.** These were Pyr05 and Pyr11 (Table 1, Fig. S2). Addition of zinc fully  
95 restored growth of *A. fumigatus* in the presence both these compounds (Fig. S3, S4). Copper  
96 was also able to fully restore growth in the presence of both compounds, while iron and  
97 manganese were able to partially restore growth in the presence of Pyr05 (Fig. S3). This  
98 indicated that the compounds affected both copper and zinc metabolism. A little growth was  
99 observed upon incubating conidia for 8 h in medium containing either of the two  
100 compounds followed by a 7 h incubation in medium without the compounds, while  
101 incubating conidia for 8 h in medium free of the compounds followed by 7 h in medium  
102 containing the compounds resulted in almost complete inhibition (Fig. S5). This suggested  
103 that the two pyrazolones were acting at the early germination stage.

104 **Porphyrin family.** These were Por06 and Por07 (Table 1, Fig S2). Addition of zinc restored  
105 growth in all but the highest tested concentrations for both compounds (Fig. S6, S7), while  
106 copper and manganese were able to partially restore growth for both (Fig. S6). Incubating  
107 conidia for 8 h in medium containing either of the two compounds followed by 7 h  
108 incubation in medium without the compounds or incubating conidia for 8 h in medium free  
109 of the compounds followed by 7 h in medium containing the compounds resulted in high  
110 levels of *A. fumigatus* inhibition (Fig. S8). This suggested that both compounds had a fast  
111 fungicidal effect. *In vitro* combination tests of Por06 resulted in indifferent results with all 3  
112 antifungal drugs (Table 2). In addition, the administration of Por06 to infected mice resulted

113 in no significant difference in survival compared to the control group. Thus, 5 out of 10 mice  
114 receiving 7.5 mg/kg/day ( $p = 0.4459$ ) and 6 out of 10 mice receiving 11.25 mg/kg/day ( $p =$   
115  $0.4004$ ) of Por06 survived compared to 12 out of 30 for the control group (Fig. S9). There  
116 was also no significant difference in luminescence between the groups (Fig. S9) and all three  
117 groups appeared similar (Fig. S10). Use of higher concentrations of Por06 to treat infected  
118 mice was not attempted, as 15 mg/kg/day resulted in 50% mortality when administered to  
119 uninfected mice.

120 **Polyaminocarboxylate family.** These were Ami03 and Ami04 (7) (Table 1, Fig S2). Addition of  
121 zinc fully restored growth in the presence both these compounds, while manganese was  
122 able to partially restore growth in the presence of Ami04 (Fig. 1, 2). The incubation of conidia  
123 for 8 h in medium containing the compounds followed by a 7 h incubation in medium  
124 without the compounds resulted in reduced growth inhibition of *A. fumigatus* conidia  
125 compared to having the compounds present throughout the incubation (Fig. 3). A reduction  
126 on inhibition was also observed when conidia were incubated for 8 h in the absence of the  
127 compounds followed by 7 h in their presence (Fig 3). This suggested that these compounds  
128 were relatively slow-acting and had a fungistatic effect on fungal growth. *In vitro*  
129 combination tests of Ami04 gave indifferent results with all 3 antifungal drugs (Table 2).  
130 Furthermore, *in vivo* experiments showed that a dose of 7.5 mg/kg/day of the  
131 polyaminocarboxylate compound Ami04 did not result in a significant difference, as only 5  
132 out of 9 mice survived ( $p = 0.4264$ ) and there was no significant difference in luminescence  
133 between the control group and the treated groups (Fig. 4, 5). In contrast, a dose of Ami04 of  
134 15 mg/kg/day was able to improve significantly the survival of immunosuppressed mice  
135 suffering from pulmonary aspergillosis ( $p = 0.0024$ ), since 10 out of 10 infected mice  
136 recovered compared to 12 out of 30 for the control group (Fig. 4). Though not statistically

137 significant; there was a 46% and a 52% reduction in luminescence on day 3 and day 5  
138 respectively in the group receiving 15 mg/kg/day of Ami04 compared to the control.

139 Lung sections from control mice displayed typical invasive aspergillosis lesions with small  
140 necrotic foci (Fig 6A), destruction of bronchiole epithelium (Fig 6BC), blood vessel invasion  
141 by the fungus (Fig 7DE), and multifocal abscesses containing hyphae (Fig 6F). In contrast,  
142 most treated mice displayed minimal to mild inflammatory lesions (Fig 6G), characterized by  
143 perivascular lymphocyte and plasma cell infiltrates (Fig 6H) with no fungi invading the  
144 parenchyma (Fig 6I). A few mice displayed randomly distributed inflammatory (with  
145 neutrophils) or necrotic lesions (Fig 6JK), with few intralesional fungi (Fig 6L).

146 In summary, these results indicated that one polyaminocarboxylate compound (Ami04)  
147 significantly improved the survival of mice suffering invasive pulmonary aspergillosis.

148

## 149 **DISCUSSION**

150 Zinc chelators have been shown to inhibit *A. fumigatus* growth (26). The 6 compounds  
151 identified by our protocol fell into 3 chemical families and each family included two analogs.  
152 This supports the reliability of our approach, since it is very unlikely that structurally similar  
153 compounds would get selected by chance. Pyrazolones, porphyrins and  
154 polyaminocarboxylates are all known to be metal ion chelators and include zinc chelators.

155 Pyrazonoles have previously demonstrated antifungal activity against *Aspergillus in vitro* (25,  
156 35) and there are pyrazolones which are known to bind to zinc to form complexes (28, 41).  
157 The two pyrazolones we identified proved effective *in vitro*, however they were structurally  
158 similar to pyrazolones found to be metabolically unstable when tested in rat liver



159 microsomes (12), which led us to focus on the other compounds we identified in our *in vivo*  
160 assays.

161 Certain porphyrins show strong selectivity towards zinc (27) and have been used as metal  
162 chelators (20, 44). The porphyrin Por06 (6) proved effective against fungi *in vitro*. Por06 had  
163 relatively low cytotoxicity against HeLa cells; however 15 mg/kg/day proved toxic when  
164 administered to mice. The lower concentrations of 7.5 and 11.25 mg/kg/day did not improve  
165 survival rates of infected mice compared to the ones receiving a placebo. Por06 fulfills only  
166 two of Lipinski's rules for determining if a compound is drug-like (29)} as it has few hydrogen  
167 bond donors and acceptors, but has a high molecular weight and logP value. The low  
168 solubility in water and the high molecular weight may therefore make it difficult for Por06 to  
169 reach the lungs from the peritoneal cavity. Another possible explanation for its lack of effect  
170 may be that it is rapidly degraded or cleared within the host body. The porphyrins identified  
171 by this study are therefore effective *in vitro*, however the one we tested *in vivo* was not able  
172 to inhibit fungal growth when administered at concentrations that are not toxic to mice.  
173 However it is possible that this porphyrin would be effective at that concentration if used in  
174 combination with other antifungal drugs.

175 Polyaminocarboxylates are commonly used in biological studies as metal chelators (10). One  
176 such compound is ethylenediaminetetraacetic acid (EDTA) (34), which binds strongly to  
177 calcium, zinc and magnesium and is able to inhibit metalloenzymes by rapidly capturing  
178 metal ions that spontaneously dissociate from them (5, 7). Polyaminocarboxylates have  
179 attracted interest as potential antimicrobial (17) or antitumor drugs (13, 23) due to their  
180 chelating activity. EDTA has proven effective *in vitro* as an antifungal agent either alone (39)  
181 or in combination with other compounds (1, 36, 37). EDTA has low toxicity since mice can

182 tolerate doses of 75 mg/kg/day (33) and it was able to reduce the mortality of rats suffering  
183 from pulmonary aspergillosis either administered alone or in combination with amphotericin  
184 B (19).

185 The polyaminocarboxylate Ami04 was able to significantly improve mouse survival in our  
186 model of invasive pulmonary aspergillosis at a dose of 15 mg/kg/day as evidenced by the  
187 survival curve and the lung sections. This molecule proved more effective than EDTA, as  
188 EDTA required a dose of 30 mg/kg/day to improve survival in an invasive aspergillosis rat  
189 model (19). In addition, our molecule had greater specificity towards zinc compared to EDTA,  
190 as it does not bind to magnesium. Moreover, the Ami04 compound did not show any toxicity  
191 towards HeLa cells or mice in the concentrations tested: Ami04 is less toxic than chelators  
192 that previously proved effective on infected mice such as TPEN or phenanthroline, (26).  
193 Ami04 fulfills all but one of Lipinski's rules for determining if a compound is drug-like (29) as  
194 it has a low molecular weight, few hydrogen bond donors and a low logP value.

195 The probable mode of action of this polyaminocarboxylate is to sequester free zinc outside  
196 the fungal cells and thus prevent them from acquiring the ions. It seems unlikely that it is  
197 able to enter the fungal cells, since other polyaminocarboxylates such as EDTA or DTTA are  
198 unable to cross cell membranes (24). This would explain why Ami03 and Ami04 primarily  
199 displayed a fungistatic effect. An inability to enter cells might also be the cause of the lower  
200 toxicity of polyaminocarboxylates compared to TPEN and phenanthroline in mammalian  
201 cells, so this could be advantageous as it results in reduced host toxicity. When tested in  
202 combination with established antifungal drugs *in vitro* Ami04, like Por06, had an indifferent  
203 effect, presumably because the mode of action of this compound is different from that of  
204 caspofungin, voriconazole and amphotericin B. However, since there is no negative

205 interaction between the drugs and our polyaminocarboxylate, it seems probable that they  
206 could be used in combination to produce an additive effect and to achieve higher survival  
207 rates *in vivo*.

208 In conclusion, our strategy aimed at selecting compounds that specifically interfere with zinc  
209 metabolism was able to identify one compound that was effective *in vivo*. This  
210 polyaminocarboxylate did not show toxicity towards cell cultures or mice at the tested  
211 concentrations. Further investigation of this compound could potentially lead to the  
212 development of novel antifungal treatment options either as a monotherapy or in  
213 combination with existing drugs.

214

## 215 MATERIALS AND METHODS

216 **Construction of strain used in this study.** The strains of *Aspergillus fumigatus* used in this  
217 study were AF14<sup>LUC</sup> (wt [PgdpA → luc<sup>cds</sup>]) and AF721<sup>LUC</sup> ( $\Delta zrfA\Delta zrfB\Delta zrfC$  [PgdpA → luc<sup>cds</sup>]).  
218 Unlike their relative strains AF14 (wt) (43), and AF721 ( $\Delta zrfA\Delta zrfB\Delta zrfC$ ) (3), the AF14<sup>LUC</sup> and  
219 AF721<sup>LUC</sup> strains were able to express constitutively at a high level a codon-optimized version  
220 of the firefly luciferase (*luc*) under control of the glyceraldehyde-3-phosphate  
221 dehydrogenase promoter (*PgdpA*) from *A. fumigatus*.

222 To construct the AF14<sup>LUC</sup> and AF721<sup>LUC</sup> bioluminescent strains we transformed both the  
223 CEA17 and AF2511 uridine-uracil-auxotrophic *pyrG1* strains (2) with a EcoRI-SphI 4777-bp  
224 DNA fragment excised from plasmid pLUC-pyrG-D (Fig. S11), which was generated by ligating  
225 a XbaI-XbaI DNA fragment (2619 bp) obtained from plasmid PgpdAAf:LucOPTAf\_ptrA (kindly  
226 provided by Dr. Matthias Brock) (18) into the only XbaI site of pPYRGQ3 plasmid (2). Since  
227 the pPYRGQ3 plasmid had been designed previously to target specifically the introduction of

228 any foreign DNA fragment between the AFUA\_2G08360 (*pyrG*) and AFUA\_2G08350 loci of  
229 any auxotrophic *A. fumigatus* strain (2), both strains harbored the [*PgdpA* → *luc*]  
230 construction inserted into the same locus, which allowed to compare the luminescence  
231 produced by these strains and, hence, to measure very accurately the fungal growth  
232 capacities of these strains.

233 **Preparation of conidial suspensions.** Conidia were harvested from the AF14<sup>LUC</sup> and AF721<sup>LUC</sup>  
234 strains. Cultures were grown for 7 days on 2% malt agar slants and recovered by vortexing  
235 with 0.01% aqueous Tween 20 (VWR International) solution. Homogenous conidial  
236 suspensions were collected following filtration through a 40 μm pore size filter (Falcon) (26).

237 **Chemical library screening.** The medium for our resazurin assay consisted of 70% v/v RPMI  
238 1640 (1X) without phenol red (Thermo Fisher Scientific), 30% v/v sterile water, 0.07% v/v  
239 TWEEN 20 (VWR International), 0.00002% w/v resazurin sodium salt, 10 μM FeSO<sub>4</sub> (Merck  
240 Millipore), 2 μM CuSO<sub>4</sub> (Merck Millipore), 2 μM MnSO<sub>4</sub> (Merck Millipore). This medium was  
241 inoculated with either 8x10<sup>4</sup> conidia/ml of AF14 with no additional ZnSO<sub>4</sub> or with 8x10<sup>4</sup>  
242 conidia/ml of AF721 with an additional 100 μM of ZnSO<sub>4</sub> (Merck Millipore). This ZnSO<sub>4</sub>  
243 concentration was sufficiently high to allow the zinc necessary for normal growth to diffuse  
244 through the cell membrane without the need for transporters. The salt solutions were made  
245 using sterile water. Using a Tecan Freedom EVO 200 platform, 130 μL of these mixtures were  
246 added to 96-well plates (F-bottom, clear, bar-coded tissue-culture plates; Greiner Bio-One):  
247 each well was previously spiked with 1 μl of compound in DMSO except columns 1 and 12  
248 dedicated to controls. Amphotericin B dissolved in DMSO was used as negative controls to  
249 kill all cells, while DMSO was used as positive controls to define 100% growth. The plates  
250 were incubated for 38 to 40 h in a 5% CO<sub>2</sub> incubator at 37° C. Then a dual-wavelength

251 measurement was performed (measurement wavelength 570 nm and reference wavelength  
252 604 nm) using a Tecan Infinite M1000 Pro microplate reader (15). For each plate, the Z'-  
253 factor (47) was calculated based on positive and negative controls and all values were above  
254 the threshold considered as an excellent assay (average Z'-factor:  $0.793 \pm 0.120$  for AF14 and  
255  $0.929 \pm 0.029$  for AF721). The data were normalized as percentage of viability relative to  
256 positive and negative controls using the following formula: % viability =  $100 \times (\text{sample value}$   
257  $- \text{average value of negative controls}) / (\text{average value of positive controls} - \text{average value of}$   
258  $\text{negative controls})$ . Compounds that caused less than 70% viability of AF14 with no additional  
259 zinc but more than 95% viability of AF721 in the presence of zinc were considered as hits and  
260 selected for further experiments.

261 **Resazurin dilution series assay.** The medium and strains ( $8 \times 10^4$  conidia/ml) in these assays  
262 were the same as those used in the library screen, except that this assay used 130  $\mu\text{l}$  of  
263 medium per well(15). The compound concentrations used in the dilution series were 100,  
264 50, 25, 12.5, 6.25, 3.1, 1.6, 0.8, 0.4 and 0.2  $\mu\text{M}$  of compound in addition to positive controls  
265 containing 1  $\mu\text{l}$  DMSO and negative controls containing 1  $\mu\text{l}$  of DMSO with 2  $\mu\text{g}$  amphotericin  
266 B. The plates were incubated in a 5%  $\text{CO}_2$  incubator at 37° C for 42 h for the resazurin assay.  
267 Each concentration was tested in duplicate, as were the plates, resulting in four total  
268 replicates. Measurements on the resazurin plates were performed as previously described.

269 **Luciferin dilution series assays.** The medium and strains ( $8 \times 10^4$  conidia/ml) in these assays  
270 were the same as those used in the library screen, except that this assay used 65  $\mu\text{l}$  of  
271 medium with no resazurin (26). The compound concentrations used in the dilution series  
272 were 100, 50, 25, 12.5, 6.25, 3.1, 1.6, 0.8, 0.4 and 0.2  $\mu\text{M}$  of compound in addition to  
273 positive controls containing 1  $\mu\text{l}$  DMSO and negative controls containing 1  $\mu\text{l}$  of DMSO with 2

274  $\mu\text{g}$  amphotericin B. The plates were incubated in a 5%  $\text{CO}_2$  incubator at 37° C for 15 h. Each  
275 concentration was tested in duplicate, as were the plates, resulting in four total replicates.  
276 Plates had 5  $\mu\text{l}$  phosphate-buffered saline (PBS) containing 4.3  $\mu\text{g}$  of D-luciferin added to  
277 each well and plates were incubated for 10 min prior to luminescence acquisition on an IVIS  
278 100 system (PerkinElmer, Boston, MA). Bioluminescence images were analyzed and the light  
279 emission (total photons flux per second) from a region of interest (ROI) quantified with  
280 Living Image software (version 3.1; PerkinElmer) (26). The percent growth at each  
281 concentration was calculated using the (Sample well/Positive control average) x100  
282 equation.

283 **Hyphal measurement and luciferin assay.** To get more precise results, compounds  
284 demonstrating an effect in luciferin dilution series assays were tested on AF14 and AF721 as  
285 in the luciferase dilution series assay except that this assay used 24-well plates with 500  $\mu\text{l}$  of  
286 medium per well seeded with  $5 \times 10^4$  conidia. Plates were incubated for 10 h at 37°C, at  
287 which point photographs were taken using an EVOS Core microscope (Thermo Fisher  
288 Scientific, Waltham, MA) at a magnification of x 20. The ImageJ software was used to  
289 measure the lengths of 100 hyphae for each sample, using the freehand line tool to trace the  
290 hyphae from the conidium to the tip of the longest hypha (26).

291 The plates were then incubated for an additional 5 h at 37°C, and luminescence  
292 measurements were taken as described in the previous section except that each well  
293 received 5  $\mu\text{l}$  phosphate-buffered saline (PBS) containing 0.16 mg of D-luciferin. Experiments  
294 were repeated twice for each concentration, and cultures were made in triplicate (18).  $\text{MIC}_{95}$   
295 (Minimum Inhibitory Concentration) was defined as the lowest concentration of a  
296 compound tested sufficient to cause at least 95% reduction in *A. fumigatus* bioluminescence

297 compared to the positive control that received no treatment, while MIC<sub>50</sub> was the minimum  
298 concentration tested able to cause at least 50% reduction.

299 The assays for measuring the effects of other ions on the compounds were identical with the  
300 zinc assay, with the exception that the 100 μM of ZnSO<sub>4</sub> was replaced with 100 μM of CuSO<sub>4</sub>,  
301 FeSO<sub>4</sub>, MgSO<sub>4</sub> or MnSO<sub>4</sub>.

302 **Fungal growth phase luciferin assay:** The effects of a short early conidial exposure to the  
303 compounds was determined by adding the compounds at the start of the incubation and  
304 removing them after 8 h by centrifuging the plate to pellet the conidia and washing the  
305 plates twice before adding fresh medium and continuing the incubation for 7 h followed by  
306 luminescence measurements. The effects of the compounds at later conidial growth stages  
307 were examined by adding the compounds after an 8 h incubation, at which point the conidia  
308 start to germinate, and then continuing the incubation for 7 h followed by luminescence  
309 measurements (26).

310 ***In vitro* combination treatment assay.** This procedure used the same medium and  
311 incubation conditions as the luciferase assay. It was performed on the Por06, Por07, Ami03  
312 and Ami04 compounds. The interactions between established antifungal drugs and library  
313 compounds were measured using the fractional inhibitory concentration index (FICI) via a  
314 checkerboard method (48). Caspofungin, amphotericin B and voriconazole were selected  
315 because they are representatives of different classes of established antifungal drugs and  
316 their mode of action does not involve zinc metabolism. The dilution series for the selected  
317 molecules was 24, 18, 12, 6, 2.4 and 0 μM, while that for the antifungal drugs was 0.1, 0.75,  
318 0.50, 0.25, 0.1 and 0 μg/ml. A 50% inhibition was employed as an endpoint for assays  
319 involving caspofungin, as it is cytostatic rather than cytotoxic and cannot achieve high levels

320 of inhibition (30), and a 90% inhibition for assays involving amphotericin B and voriconazole.  
321 The FICI was defined as  $(Ac/Aa) + (Bc/Ba)$ , where Ac and Bc are the endpoint values of the  
322 library compound and antifungal drug in combination, Aa is the endpoint value of the library  
323 compound alone, and Ba is the endpoint value of the antifungal alone. Interactions were  
324 classified as synergistic (FICI of  $\leq 0.5$ ), indifferent (FICI of  $>0.5$  but  $\leq 4$ ), or antagonistic (FICI of  
325  $>4$ ) (26).

326 **HeLa cell cytotoxicity assay.** Analysis of toxicity to human cells was performed as previously  
327 described (38) using the Cytotoxicity Detection Kit (LDH) (Roche) according to manufacturer's  
328 instructions. This assay measures the activity of lactate dehydrogenase in a culture's  
329 supernatant to estimate percent cytotoxicity. It was performed on the compounds selected  
330 by the luciferin dilution series assay. Briefly, 100  $\mu\text{l}$  of a  $5 \times 10^5$  cells/ml suspension in PAA  
331 Quantum 286 Complete Epithelial Medium (Brunschwig Chemicals) with L-glutamine,  
332 penicillin and streptomycin but no serum was placed in the wells of a 96 well plate and left  
333 to grow overnight at 37°C with 10% CO<sub>2</sub>. The supernatant was replaced with 200  $\mu\text{l}$  of fresh  
334 medium containing 10  $\mu\text{M}$  or 100  $\mu\text{M}$  of library compound in the sample wells, 1% TritonX-  
335 100 in the positive control wells, nothing in the negative control wells, while the background  
336 control consisted solely of 200  $\mu\text{l}$  of medium. All samples and controls were in triplicate. The  
337 cells were left to grow for 24 h at 37°C with 5% CO<sub>2</sub>. 100  $\mu\text{l}$  of culture supernatant from each  
338 well was transferred to a new 96 well plate to which 100  $\mu\text{l}$  of reaction mixture containing  
339 the dye iodonitrotetrazolium was added and the plate was incubated for 0.5 h. Absorbance  
340 was measured at 492 nm and 604 nm using a Dynex ELISA Processor (Magellan Biosciences)  
341 and the percent cytotoxicity was calculated using the equation  $100 \times (\text{mean of sample}$   
342  $\text{triplicates} - \text{negative control}) / (\text{positive control} - \text{negative control})$ .



343 **Murine toxicity assays and infection.** In this procedure we used our model of invasive  
344 pulmonary aspergillosis (26) with male BALB/CJ mice (23 to 28 g, 8 weeks old) supplied by  
345 the R. Janvier breeding center (Le Genest Saint-Isle, France). Mice were cared for in  
346 accordance with Institut Pasteur guidelines, in compliance with European animal welfare  
347 regulation. This study was approved by the ethical committee for animal experimentation  
348 (Comité d'Éthique en Experimentation Animale [CETEA], project license number 2013-0020).  
349 At 4 days and 1 day before the start of a toxicity assay or of infection, each mouse received  
350 an immunosuppressive regimen by intraperitoneal (i.p.) injection of 200  $\mu$ l  
351 cyclophosphamide (4 mg/ml). The mice remained immunosuppressed for around 7 days,  
352 which was sufficient for them to succumb to infection if left untreated. Mice used for toxicity  
353 assays received 100  $\mu$ l i.p. injections of 20% DMSO in saline solution containing the  
354 compounds on a daily basis for 10 days.

355 Mice to be infected were inoculated intranasally with a dose of  $7.5 \times 10^4$  conidia in 25  $\mu$ l of  
356 PBS containing 0.01% Tween. Following infection, the compounds or placebo were  
357 administered by i.p. injection at the indicated concentrations in a final volume of 100  $\mu$ l. The  
358 placebo consisted of 20% DMSO in saline solution. Bioluminescence imaging was started 24  
359 h after infection and was continued every other day. Images were acquired using an IVIS 100  
360 system as previously described (8). Experiments extended 14 days post-infection, including  
361 10 days of daily treatment. Infected mouse experiments were only performed on the Por06  
362 and the Ami04 small molecules, as they each represented one of the two most promising  
363 chemical families we identified and because they were the compounds of which we had  
364 sufficient amounts to perform mouse experiments.

365 **Histological analysis of Lung sections.** Mice were euthanized at day 3 post-inoculation.  
366 Lungs were immediately fixed in 4% neutral-buffered formalin and embedded in paraffin. 5  
367  $\mu\text{m}$  sections were cut and stained with Grocott's methenamine silver staining (GMS) for  
368 detection of fungi (18).

369 **Statistical analyses.** For the *in vitro* tests, the luminescence values of the different cultures  
370 in the presence of chelators and/or metal ions were compared to those of the control  
371 cultures using unpaired Student t tests with Welch's correction. Levels of significance for  
372 hyphal lengths were calculated using the Mann-Whitney test. For the *in vivo* tests, survival  
373 rates were performed by creating Kaplan-Meier plots and then performing log rank tests.  
374 Comparisons of luminescence between different mouse groups were done using an unpaired  
375 Student t test with Welch's correction. All results are expressed as means  $\pm$  standard errors  
376 of the mean (SEM), and comparisons for survival studies were considered significant if the P  
377 value was  $<0.05$ . All tests were performed using GraphPad Prism 7 software. All the  $\text{MIC}_{50}$   
378 and  $\text{MIC}_{95}$  values reported were statistically significant, and the P values indicate the level of  
379 significance compared to the positive controls (26).

380

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- 534

535

536 **TABLES**537 Table 1: Minimum inhibitory concentrations of selected compounds in  $\mu\text{g/ml}$ .  $\text{MIC}_{95}$ 538 indicates a  $\geq 95\%$  growth reduction,  $\text{MIC}_{50}$  indicates a  $\geq 50\%$  growth reduction.

<u>Compound</u>	$\text{MIC}_{95}$	$\text{MIC}_{50}$
Pyr05	13	1.3
Pyr11	6.3	2.5
Por06	14	1.4
Por07	32	3.2
Ami03	5	0.5
Ami04	20	0.4

539

540 Table 2: Interactions between established antifungal drugs and library compounds using the

541 fractional inhibitory concentration index (FICI). All results were between 0.5 and 4 indicating

542 an indifferent result and no interactions between the compounds.

<u>Compound</u>	<u>Caspofungin</u>	Voriconazole	Amphotericin B
Por06	0.6	1.0	1.2
Ami04	1.2	1.0	0.6

543

544 **FIGURE LEGENDS**545 **Figure 1.** Percent inhibition based on luminescence measurements of *A. fumigatus* wild type546 (AF14) grown either with no added ions or with the addition of  $100 \mu\text{M}$   $\text{CuSO}_4$ ,  $\text{FeSO}_4$ ,

547 MgSO<sub>4</sub>, MnSO<sub>4</sub> or ZnSO<sub>4</sub> for 15 h in the presence of the polyaminocarboxylate (A) Ami03 or  
548 (B) Ami04.

549 **Figure 2.** Hyphal length percent inhibition of *A. fumigatus* wild type (AF14) or triple zinc  
550 transporter knockout (AF721) grown either with no added zinc or with 100 μM ZnSO<sub>4</sub> for 10  
551 h in the presence of the polyaminocarboxylate (A) Ami03 or (B) Ami04.

552 **Figure 3.** Percent inhibition based on luminescence measurements of *A. fumigatus* wild type  
553 (AF14) grown in the presence of the polyaminocarboxylate (A) Ami03 or (B) Ami04. Removed  
554 at 8 h: medium was replaced with fresh medium containing no tested compound after an 8 h  
555 incubation. Added at 8 h: compounds were added to the medium after 8 h of incubation.  
556 The cultures were incubated for an additional 7 h, resulting in a total incubation time of 15  
557 h.

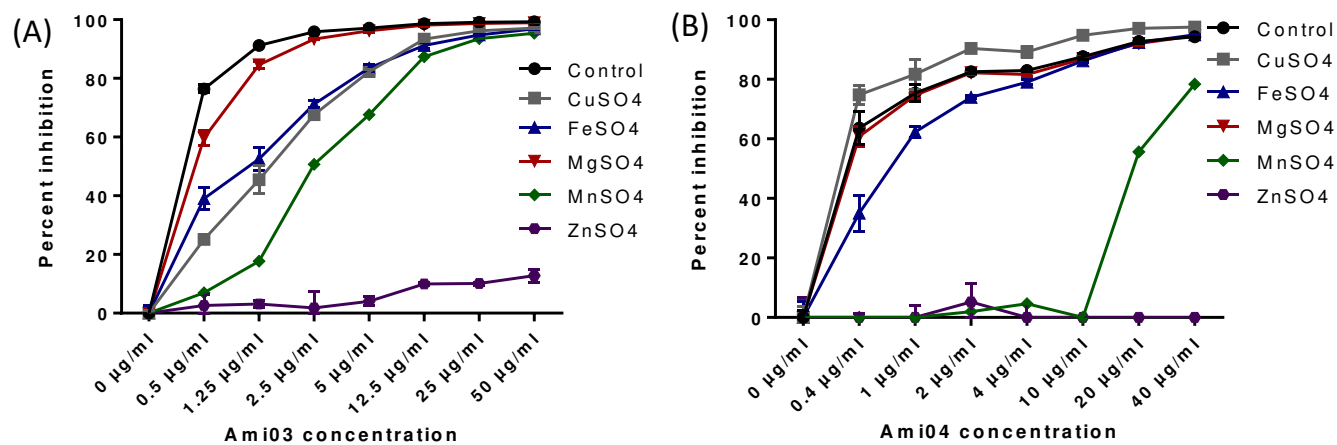
558 **Figure 4.** (A) Percent survival and (B) luminescence of immunosuppressed mice that were  
559 intranasally infected with  $7.5 \times 10^4$  *A. fumigatus* wild type (AF14) conidia and treated with  
560 the polyaminocarboxylate Ami04. 15 mg/kg/day was able to significantly improve mouse  
561 survival ( $p = 0.0024$ ) and resulted in a 46% reduction in luminescence on day 3 and 52%  
562 reduction on day 5 compared to the control group.

563 **Figure 5.** Examples showing luminescence of mice treated with 7.5 or 15 mg/kg/day of the  
564 polyaminocarboxylate Ami04 and of a DMSO placebo group. Mice in all three groups  
565 developed aspergillosis, however only the ones receiving 15 mg/kg/day showed 100%  
566 survival.

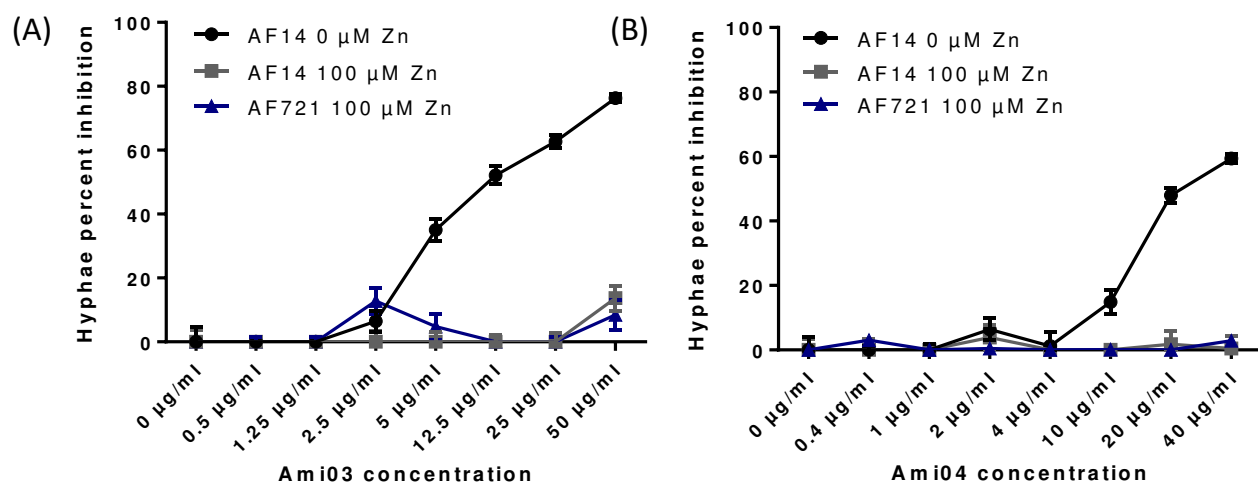
567 **Figure 6:** Treated mice displayed less severe lung invasion by the fungus. Control mice  
568 displayed very heterogeneous lesions: from (A) small necrotic foci (black arrowhead), with  
569 (B,C) destruction of bronchiole epithelium (black arrowhead) and blood vessel invasion by

570 the fungus (DE), to randomly distributed multifocal abscesses containing hyphae (F). Most  
571 treated mice (6/10) displayed minimal to mild inflammatory lesions, characterized by  
572 perivascular lymphocyte and plasma cell infiltrates (black arrowheads) (GH), with no fungi  
573 invading the parenchyma (I). Less frequently mice (4/10), displayed randomly distributed  
574 inflammatory (with neutrophils) (JK) or necrotic lesions (black arrowhead), with few  
575 intralesional fungi (black arrowhead) (L). A, B, D, E, G, H, J, K: HE staining; C, F, I, L: Gomori  
576 Grocott staining.

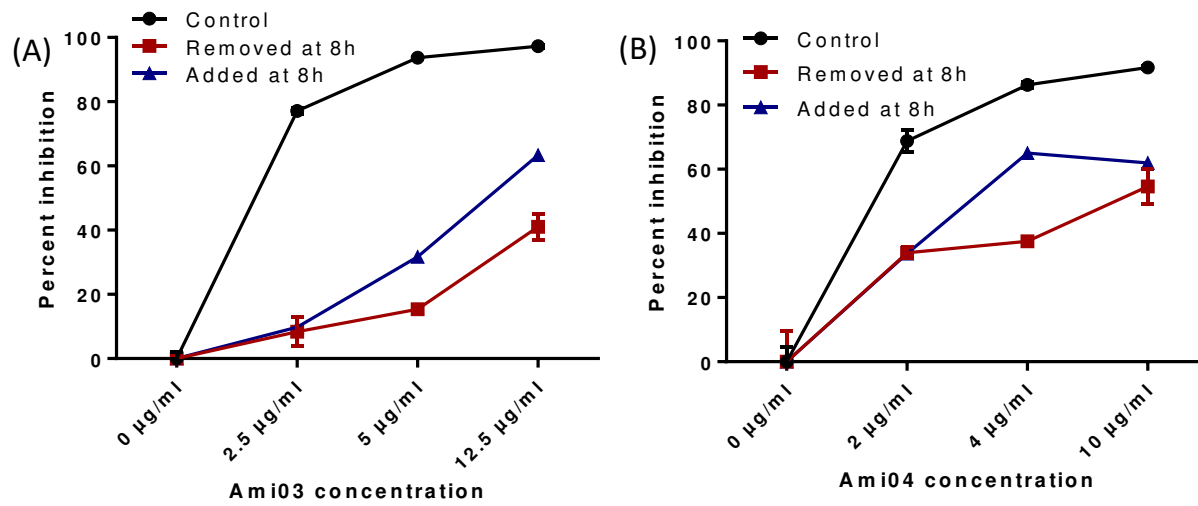




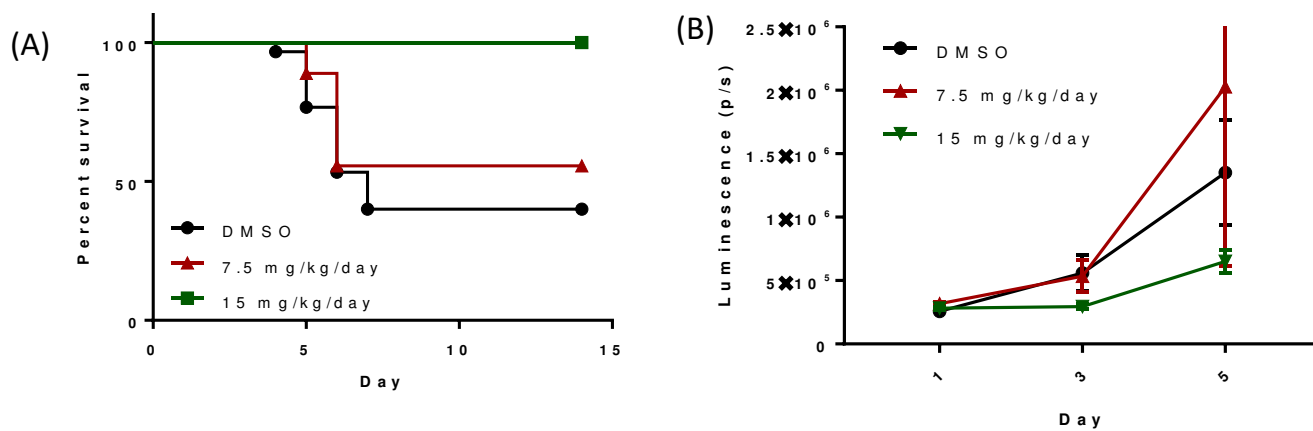
**Figure 1.** Percent inhibition based on luminescence measurements of *A. fumigatus* wild type (AF14) grown either with no added ions or with the addition of 100  $\mu$ M CuSO<sub>4</sub>, FeSO<sub>4</sub>, MgSO<sub>4</sub>, MnSO<sub>4</sub> or ZnSO<sub>4</sub> for 15 h in the presence of the polyaminocarboxylate (A) Ami03 or (B) Ami04.



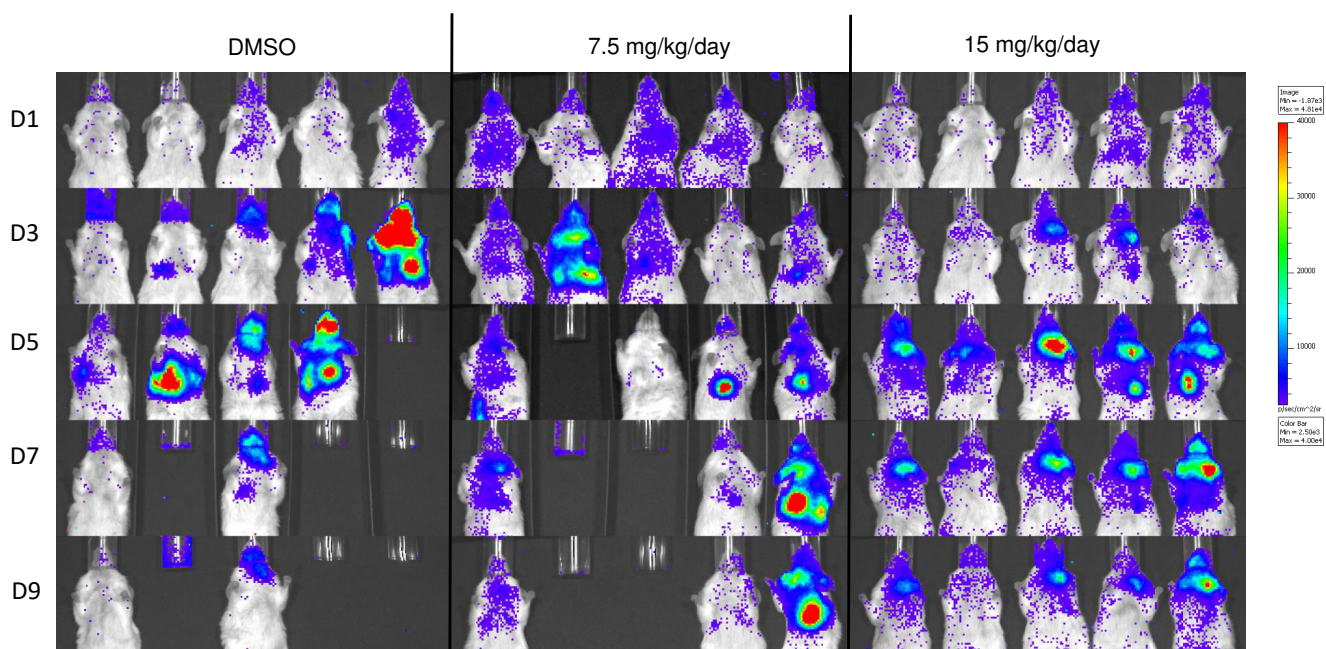
**Figure 2.** Hyphal length percent inhibition of *A. fumigatus* wild type (AF14) or triple zinc transporter knockout (AF721) grown either with no added zinc or with 100 μM ZnSO<sub>4</sub> for 10 h in the presence of the polyaminocarboxylate (A) Ami03 or (B) Ami04.



**Figure 3.** Percent inhibition based on luminescence measurements of *A. fumigatus* wild type (AF14) grown in the presence of the polyaminocarboxylate (A) Ami03 or (B) Ami04. Removed at 8 h: medium was replaced with fresh medium containing no tested compound after an 8 h incubation. Added at 8 h: compounds were added to the medium after 8 h of incubation. The cultures were incubated for an additional 7 h, resulting in a total incubation time of 15 h.



**Figure 4.** (A) Percent survival and (B) luminescence of immunosuppressed mice that were intranasally infected with  $7.5 \times 10^4$  *A. fumigatus* wild type (AF14) conidia and treated with the polyaminocarboxylate Ami04. 15 mg/kg/day was able to significantly improve mouse survival ( $p = 0.0024$ ) and resulted in a 46% reduction in luminescence on day 3 and 52% reduction on day 5 compared to the control group.



**Figure 5.** Examples showing luminescence of mice treated with 7.5 or 15 mg/kg/day of the polyaminocarboxylate Ami04 and of a DMSO placebo group. Mice in all three groups developed aspergillosis, however only the ones receiving 15 mg/kg/day showed 100% survival.

**Figure 6:** Treated mice displayed less severe lung invasion by the fungus. Control mice displayed very heterogeneous lesions: from (A) small necrotic foci (black arrowhead), with (B,C) destruction of bronchiole epithelium (black arrowhead) and blood vessel invasion by the fungus (DE), to randomly distributed multifocal abscesses containing hyphae (F). Most treated mice (6/10) displayed minimal to mild inflammatory lesions, characterized by perivascular lymphocyte and plasma cell infiltrates (black arrowheads) (GH), with no fungi invading the parenchyma (I). Less frequently mice (4/10), displayed randomly distributed inflammatory (with neutrophils) (JK) or necrotic lesions (black arrowhead), with few intralesional fungi (black arrowhead) (L). A, B, D, E, G, H, J, K: HE staining; C, F, I, L: Gomori Grocott staining.

