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

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ABSTRACT

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

INTRODUCTION

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

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

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

Calprotectin, which has a zinc chelating activity, has been used to inhibit the growth of *A. fumigatus* in the corneas of immunocompetent mice (14). In addition, our group has used 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±3.5 µM, only 116 compounds were found to inhibit the growth
83 of *A. fumigatus* wild type ($AF14^{LUC}$) with no added zinc, but permit the growth of the *A.*
84 *fumigatus* triple zinc transporter knockout ($AF721^{LUC}$) with 100 µM 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 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

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

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

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

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

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

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

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

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

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

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

MATERIALS AND METHODS

Construction of strain used in this study. The strains of *Aspergillus fumigatus* used in this study were AF14^{LUC} (wt [P*gdpA* → *luc*^{cds}]) and AF721^{LUC} (Δ*zrfA*Δ*zrfB*Δ*zrfC* [P*gdpA* → *luc*^{cds}]). Unlike their relative strains AF14 (wt) (43), and AF721 (Δ*zrfA*Δ*zrfB*Δ*zrfC*) (3), the AF14^{LUC} and AF721^{LUC} strains were able to express constitutively at a high level a codon-optimized version of the firefly luciferase (*luc*) under control of the glyceraldehyde-3-phosphate dehydrogenase promoter (P*gdpA*) from *A. fumigatus*.

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

any foreign DNA fragment between the AFUA_2G08360 (*pyrG*) and AFUA_2G08350 loci of any auxotrophic *A. fumigatus* strain (2), both strains harbored the [P*gdpA* → *luc*] construction inserted into the same locus, which allowed to compare the luminescence produced by these strains and, hence, to measure very accurately the fungal growth capacities of these strains.

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

Chemical library screening. The medium for our resazurin assay consisted of 70% v/v RPMI 1640 (1X) without phenol red (Thermo Fisher Scientific), 30% v/v sterile water, 0.07% v/v TWEEN 20 (VWR International), 0.00002% w/v resazurin sodium salt, 10 µM $FeSO_4$ (Merck Millipore), 2 µM $CuSO_4$ (Merck Millipore), 2 µM $MnSO_4$ (Merck Millipore). This medium was inoculated with either $8x10^4$ conidia/ml of AF14 with no additional $ZnSO_4$ or with $8x10^4$ conidia/ml of AF721 with an additional 100 µM of $ZnSO_4$ (Merck Millipore). This $ZnSO_4$ concentration was sufficiently high to allow the zinc necessary for normal growth to diffuse through the cell membrane without the need for transporters. The salt solutions were made using sterile water. Using a Tecan Freedom EVO 200 platform, 130 µL of these mixtures were added to 96-well plates (F-bottom, clear, bar-coded tissue-culture plates; Greiner Bio-One): each well was previously spiked with 1 µl of compound in DMSO except columns 1 and 12 dedicated to controls. Amphotericin B dissolved in DMSO was used as negative controls to kill all cells, while DMSO was used as positive controls to define 100% growth. The plates were incubated for 38 to 40 h in a 5% CO_2 incubator at 37° C. Then a dual-wavelength

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

Resazurin dilution series assay. The medium and strains ($8x10^4$ conidia/ml) in these assays were the same as those used in the library screen, except that this assay used 130 µl of medium per well(15). The compound concentrations used in the dilution series were 100, 50, 25, 12.5, 6.25, 3.1, 1.6, 0.8, 0.4 and 0.2 µM of compound in addition to positive controls containing 1 µl DMSO and negative controls containing 1 µl of DMSO with 2 µg amphotericin B. The plates were incubated in a 5% CO_2 incubator at 37° C for 42 h for the resazurin assay. Each concentration was tested in duplicate, as were the plates, resulting in four total replicates. Measurements on the resazurin plates were performed as previously described.

Luciferin dilution series assays. The medium and strains ($8x10^4$ conidia/ml) in these assays were the same as those used in the library screen, except that this assay used 65 µl of medium with no resazurin (26). The compound concentrations used in the dilution series were 100, 50, 25, 12.5, 6.25, 3.1, 1.6, 0.8, 0.4 and 0.2 µM of compound in addition to positive controls containing 1 µl DMSO and negative controls containing 1 µl of DMSO with 2

μg amphotericin B. The plates were incubated in a 5% CO_2 incubator at 37° C for 15 h. Each concentration was tested in duplicate, as were the plates, resulting in four total replicates. Plates had 5 μl phosphate-buffered saline (PBS) containing 4.3 μg of D-luciferin added to each well and plates were incubated for 10 min prior to luminescence acquisition on an IVIS 100 system (PerkinElmer, Boston, MA). Bioluminescence images were analyzed and the light emission (total photons flux per second) from a region of interest (ROI) quantified with Living Image software (version 3.1; PerkinElmer) (26). The percent growth at each concentration was calculated using the (Sample well/Positive control average) x100 equation.

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

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

compared to the positive control that received no treatment, while MIC_{50} was the minimum concentration tested able to cause at least 50% reduction.

The assays for measuring the effects of other ions on the compounds were identical with the zinc assay, with the exception that the 100 µM of $ZnSO_4$ was replaced with 100 µM of $CuSO_4$, $FeSO_4$, $MgSO_4$ or $MnSO_4$.

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

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

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

HeLa cell cytotoxicity assay. Analysis of toxicity to human cells was performed as previously described (38)using the Cytotoxicity Detection Kit (LDH) (Roche) according to manufacturer's instructions. This assay measures the activity of lactate dehydrogenase in a culture's supernatant to estimate percent cytotoxicity. It was performed on the compounds selected by the luciferin dilution series assay. Briefly, 100 µl of a $5x10^5$ cells/ml suspension in PAA Quantum 286 Complete Epithelial Medium (Brunschwig Chemicals) with L-glutamine, penicillin and streptomycin but no serum was placed in the wells of a 96 well plate and left to grow overnight at 37°C with 10% CO_2. The supernatant was replaced with 200 µl of fresh medium containing 10 µM or 100 µM of library compound in the sample wells, 1% TritonX-100 in the positive control wells, nothing in the negative control wells, while the background control consisted solely of 200 µl of medium. All samples and controls were in triplicate. The cells were left to grow for 24 h at 37°C with 5% CO_2. 100 µl of culture supernatant from each well was transferred to a new 96 well plate to which 100 µl of reaction mixture containing the dye iodonitrotetrazolium was added and the plate was incubated for 0.5 h. Absorbance was measured at 492 nm and 604 nm using a Dynex ELISA Processor (Magellan Biosciences) and the percent cytotoxicity was calculated using the equation 100x (mean of sample triplicates – negative control)/(positive control – negative control).

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

Mice to be infected were inoculated intranasally with a dose of $7.5x10^4$ conidia in 25 µl of PBS containing 0.01% Tween. Following infection, the compounds or placebo were administered by i.p. injection at the indicated concentrations in a final volume of 100 µl. The placebo consisted of 20% DMSO in saline solution. Bioluminescence imaging was started 24 h after infection and was continued every other day. Images were acquired using an IVIS 100 system as previously described (8). Experiments extended 14 days post-infection, including 10 days of daily treatment. Infected mouse experiments were only performed on the Por06 and the Ami04 small molecules, as they each represented one of the two most promising chemical families we identified and because they were the compounds of which we had sufficient amounts to perform mouse experiments.

Antimicrobial Agents and Chemotherapy

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 µm 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 ± 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 MIC_{50}
378 and 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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535

536 **TABLES**

537 Table 1: Minimum inhibitory concentrations of selected compounds in µg/ml. MIC_{95}

538 indicates a ≥95% growth reduction, MIC_{50} indicates a ≥50% growth reduction.

Compound	MIC_{95}	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.

Compound	Caspofungin	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 type

546 (AF14) grown either with no added ions or with the addition of 100 µM CuSO4, FeSO4,

MgSO4, MnSO4 or ZnSO4 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 ZnSO4 for 10 h in the presence of the polyaminocarboxylate (A) Ami03 or (B) Ami04.

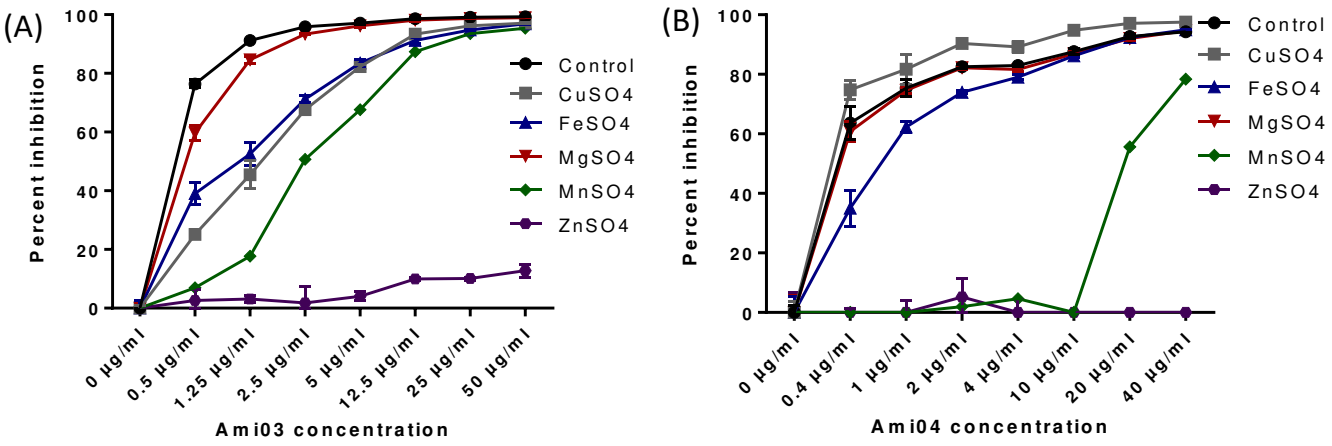
Figure 3. Percent inhibition based on luminescence measurements of *A. fumigatus* wild type (AF14) grown in the presence of the polyaminocarboxylate (A) Ami03 or (B) Ami04. Removed at 8 h: medium was replaced with fresh medium containing no tested compound after an 8 h incubation. Added at 8 h: compounds 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 7.5x10^4 *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.



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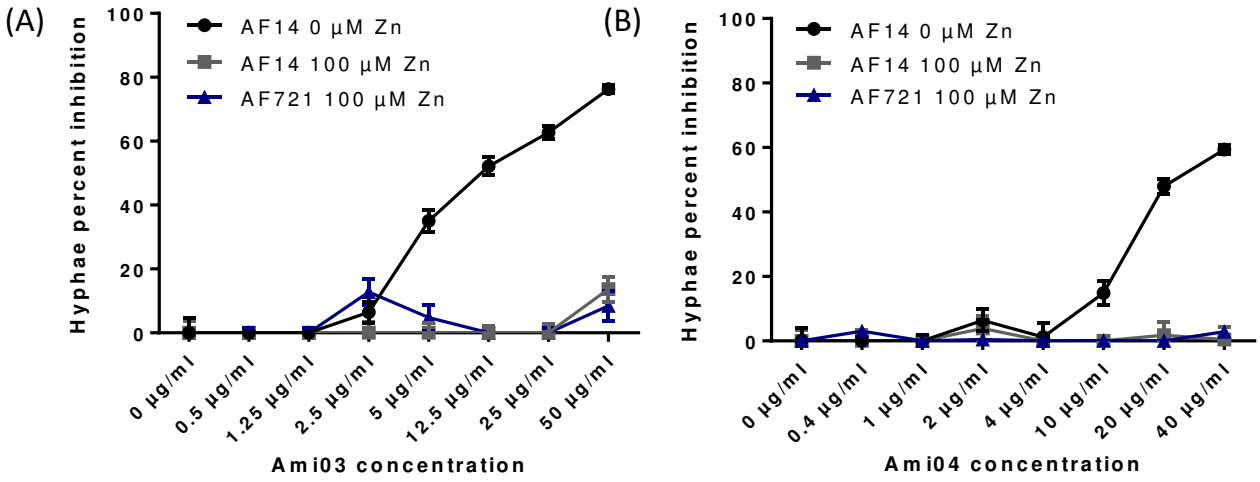

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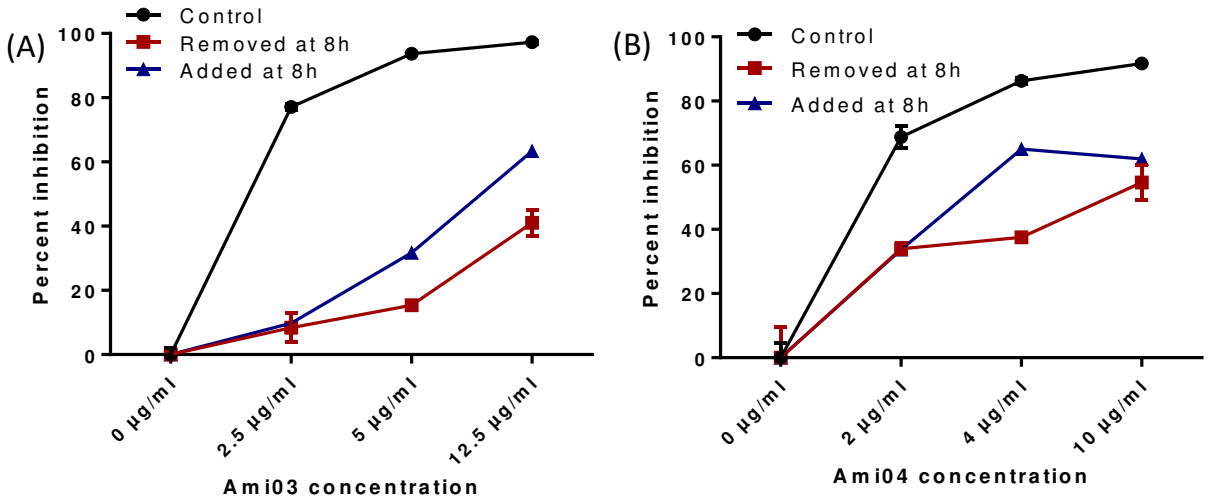

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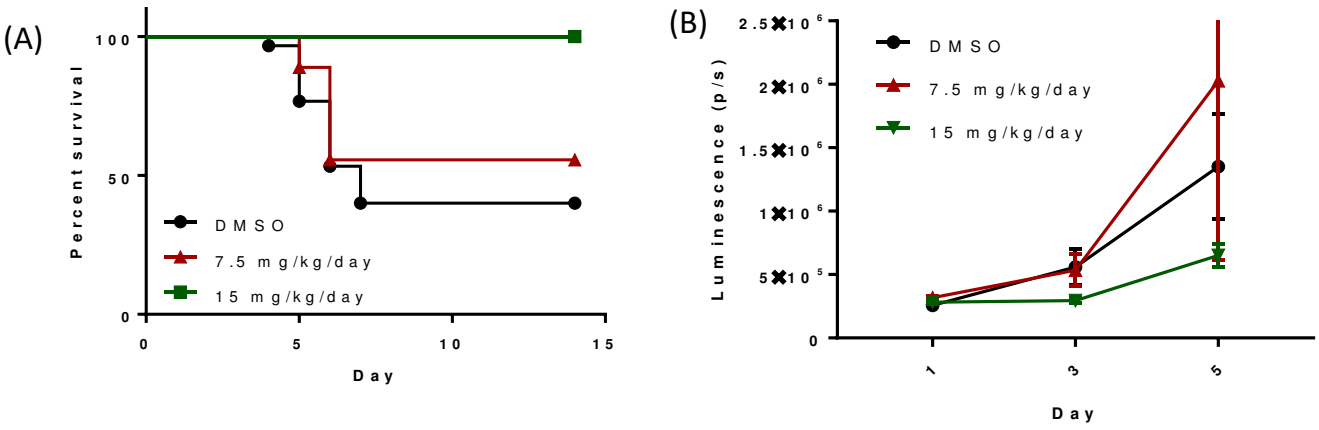

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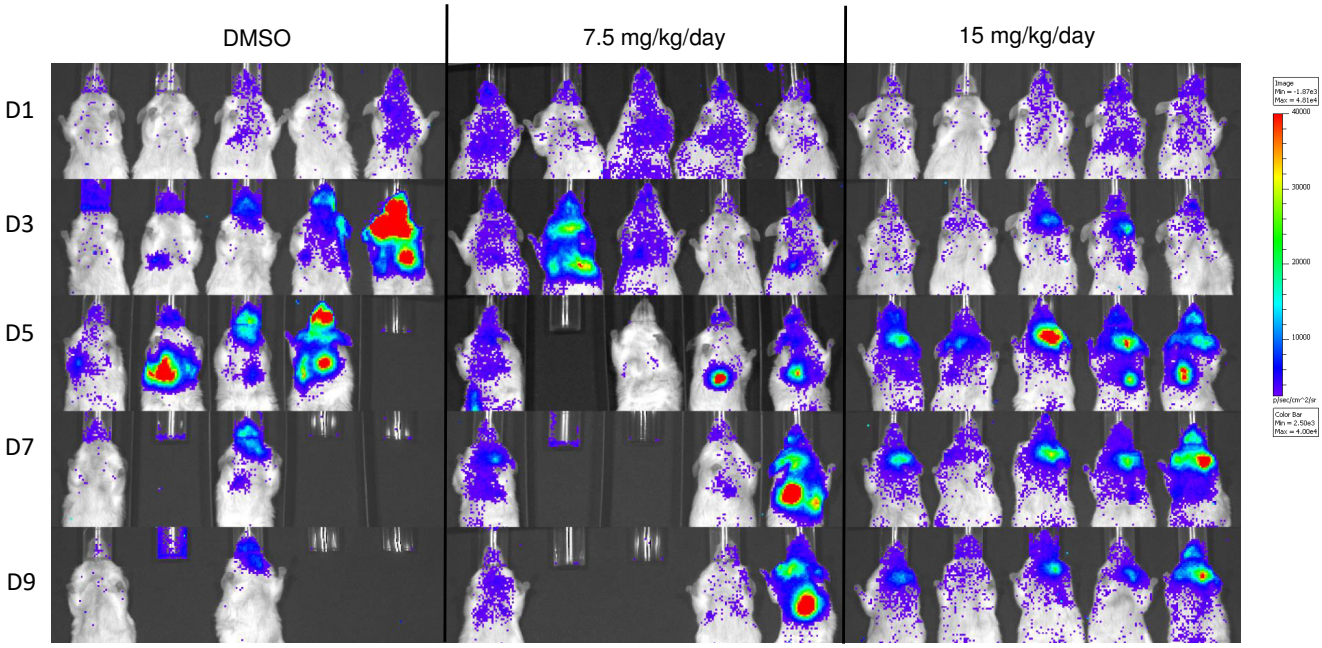

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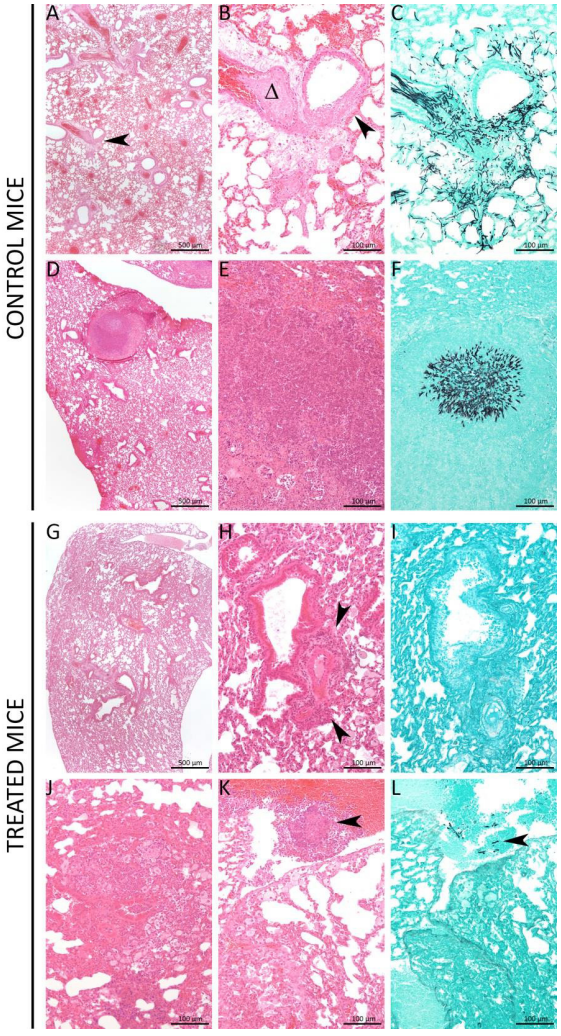

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