Abstract

Aspergillus fumigatus can infect immunocompromised patients, leading to high mortality rates due to the lack of reliable treatment options. This pathogen requires uptake of zinc from host tissues in order to successfully grow and cause virulence. Reducing the availability of that micronutrient could help treat A. fumigatus infections. In this study we examined the \textit{in vitro} effects of seven chelators using a bioluminescent strain of \textit{A. fumigatus}. 1,10-phenanthroline and TPEN (N,N,N',N'-tetrakis(2-pyridylmethyl)ethane-1,2-diamine) proved to be the most effective chelators at inhibiting fungal growth. Intraperitoneal administration of either phenanthroline or TPEN resulted in a significant improvement in survival and decrease of weight loss and fungal burden for immunosuppressed mice intranasally infected with \textit{A. fumigatus}. In vitro both chelators had an indifferent effect when employed in combination with caspofungin. The use of TPEN in combination with caspofungin also significantly increased survival compared to using these drugs individually. Our results suggest that zinc chelation may be a valid strategy for dealing with \textit{A. fumigatus} infections and that both phenanthroline and TPEN could potentially be used either independently or in combination with caspofungin, indicating that their use in combination with other antifungal treatments might also be applicable.

Introduction
Aspergillus fumigatus is a widespread soil-dwelling fungal saprotroph (1). It is one of the most ubiquitous fungal species with airborne conidia, and it is estimated that all humans inhale several hundred conidia each day (2). These are completely innocuous to immunocompetent hosts. However, they are able to develop and cause invasive pulmonary aspergillosis (IPA) in immunocompromised individuals (3). This disease is difficult to treat with a mortality rate of 45.6% (4). Commonly used drug treatment options include triazoles such as voriconazole, which inhibit ergosterol synthesis, amphotericin B, which binds to ergosterol which results in increased permeability of the cell membrane, and echinocandins such as caspofungin, which inhibit glucan synthesis (5).

Both fungal and bacterial pathogens require cations to grow within their hosts and utilize specialized mechanisms in order to obtain them (6). Zinc is considered essential for all organisms including pathogens (7). The average concentration of free Zn$^{2+}$ in human serum is 0.08 μM, which is about 150 times lower than the minimal concentration required for A. fumigatus to grow optimally in a defined liquid medium (8). A. fumigatus possesses three genes, zrfA, zrfB and zrfC, encoding plasma membrane zinc transporters (8). The zrfA and zrfB genes are required for zinc uptake in acidic Zn-limited environments (7) while the zrfC is required for zinc uptake in alkaline environments (9). The zrfC gene is primarily responsible for zinc acquisition within a host’s lungs and required for virulence; zrfA and zrfB contribute to fungal pathogenesis to a lesser extent than zrfC and are not required for virulence if zrfC is present (8). Zinc uptake is induced by the zafA transcriptional activator under Zn-limiting conditions and its deletion abrogates A. fumigatus virulence (10). Hence, by reducing the availability of zinc the
growth of *A. fumigatus* might be inhibited, which could have clinical applications as suggested recently (11). In this regard, the non-specific chelator EDTA has been successfully tested *in vitro* against *Aspergillus* (12). In addition, calprotectin, which is a zinc and manganese chelator produced by neutrophils, can inhibit the growth of *A. fumigatus* in the mouse cornea of immunocompetent mice (13). In contrast, calprotectin was not efficient in inhibiting the fungal growth in the lung abscesses of immunosuppressed mice largely due to the strong zinc scavenging capacity of ZrfC (8).

The objectives of this study were (i) to test the effects in vivo and in vitro of zinc chelators tested alone or in combination with antifungal drugs: (ii) to discover whether the chelators are fungicidal or fungistatic; and (iii) to determine the effect of hypoxia and of mammalian sera on the selected chelators;

**Materials and Methods**

The salts solutions used in this study were the following: FeSO₄, MgSO₄, MnSO₄, ZnCl₂ and ZnSO₄.

The chelators used in this study were the following:

i) Clioquinol (5-chloro-7-iido-quinolin-8-ol), a halogenated 8-hydroxyquinoline (14). It was selected because it has been extensively used as a zinc chelator both *in vitro* and *in vivo* (15-19). Clioquinol (Fluca, 33931-100MG-R) was diluted to a 0.05 M stock solution in 100% DMSO. Before use *in vitro*, the stock solution was further diluted to 5 mM in 100% DMSO and then to 0.5 mM in 10% DMSO.

ii) DEDTC (Sodium diethyldithiocarbamate trihydrate), a water-soluble organosulfur compound used as chelating agent for transition metal ions and to remove zinc from
human cell cultures (20, 21). DEDTC (Sigma-Aldrich, D3506-100G) was diluted to
a 0.1 M stock solution in dH₂O.

iii) DTPA (diethylene triamine pentaacetic acid) is a membrane-impermeable chelator
that has been employed to sequester zinc in cell cultures and is structurally similar to
EDTA (22, 23). DTPA (Sigma-Aldrich, D1133-5G) was diluted to a 0.1 M stock
solution in 0.5 N HCl.

iv) EDDA (Ethylenediamine-N,N′-diacetic acid) is an aminopolycarboxylic acid that has
been used to chelate zinc in cell cultures (24). EDDA (Sigma-Aldrich, 158186-1G)
was diluted to a 0.1 M stock solution in 0.5 N NaOH.

v) EDTA (Ethylenediaminetetraacetic acid calcium disodium salt), a water-soluble
aminopolycarboxylic acid. It was selected because it has been used as a zinc chelator
for both A. fumigatus (25) and human cells (26). EDTA (Sigma-Aldrich, ED2SC-
250G) was diluted to a 0.1 M stock solution in dH₂O.

vi) 1,10-Phenanthroline is a membrane-permeable (27) heterocyclic compound with a
strong inhibitory effect on zinc metallopeptidases (28). It has been employed as a
zinc chelator in cell cultures (29) as well as in mice (30) and rats (31).
Phenanthroline (Sigma-Aldrich, 131377-5G) 0.2 M stock solution in 100% DMSO.
Before use in vitro, the stock solution was further diluted to 0.5 mM in 1% DMSO.

vii) TPEN (N,N,N,N-Tetrakis(2-pyridylmethyl)-1,2-ethylenediamine) is a membrane-
permeable zinc chelator that has also been used extensively in that role in vivo (18,
19, 32, 33) and on A. fumigatus (25). TPEN (Life Technologies, T1210) was diluted
to a 0.05 M stock solution in 100% DMSO. Before use in vitro, the stock solution
was further diluted to 0.5 mM in 1% DMSO.
Preparation of conidial suspensions.

Conidia were produced from *A. fumigatus* strain 2/7/1, a modified version of the bioluminescent C3 strain containing a codon-optimized version of the *P. pyralis* luciferase gene. This strain behaved and caused virulence similarly to the wild type strain (34), cultures grown for 7 days on 2% malt agar slants, and recovered by vortexing with 0.01% aqueous Tween-20 solution. Homogenous conidial suspensions were collected following filtration through a 40 μm pore-size filter (Falcon) (35).

In vitro studies of chelator growth inhibition by bioluminescence detection.

*In vitro* susceptibility of the *A. fumigatus* 2/7/1 strain in liquid cultures against chelators was determined by seeding 5x10⁴ conidia in a 24-well plate. Each well contained 500 μl of RPMI 1640 cell culture medium (Invitrogen 22409-015, Gibco, France) supplemented with 10% fetal calf serum (complete RPMI) (34). For experiments with other sera 10% human, rat, mouse, rabbit serum was used to ensure that the serum environment specific to each species does not interfere individually with the properties of the chelators. These were added at different concentrations and at the time points indicated in the specific experiments. Plates were incubated for 10 h at 37°C, 5 μl PBS containing 0.16 mg of D-luciferin was then added to each well, and plates were incubated for 10 min prior to luminescence acquisition on an IVIS 100 system (PerkinElmer, Boston, MA) as previously reported (34). Photons were collected for 1 and 3 min on the high-sensitivity setting. Bioluminescence images were analyzed and the light emission (total photons flux/s) from a region of interest (ROI) quantified with Living Image software (version 3.1; PerkinElmer). Plates were incubated for an additional 5 h at 37°C and luminescence
measurements were repeated as described previously. Experiments were repeated twice for each concentration, and cultures were made in triplicate (34). The length of hyphae was measured by taking photographs on an EVOS Core microscope (Thermo Fisher Scientific, Waltham, MA) at 20x magnification, followed by measuring the lengths of 100 hyphae for each sample using the ImageJ software. The freehand line tool was used to measure from the conidium to the tip of the longest hypha (34). The MIC-0 and MIC-2 were defined as the lowest concentration of compound tested that was sufficient to result in at least 95% and 50% reduction in bioluminescence or hyphal length respectively compared to the positive control (36). The following results reflect measurements taken after 10 hours of incubation for hyphal lengths and 15 hours for luminescence. DMSO did not have a significant effect when used at the concentrations used for the chelators. The EUCAST microdilution method was used to obtain MIC and MEC values, where MIC indicates no visible growth and MEC indicates highly stunted growth, as previously described (37)(38). Briefly, 2x10⁴ conidia were seeded in a 96 well plate containing 200 μl of RPMI 1640 medium with 2% glucose per well in the presence of a chelator concentration gradient and incubated for 48 h at 37°C. The dilution range for the tested chelators was 0.065-32 mg/L and fungal growth was determined using an EVOS Core microscope at 20x magnification.

To determine the effects of chelators during a limited time period at the start of incubation, the chelators were added at 0 h, and then removed by centrifuging the plate to pellet the conidia, washing the wells twice before adding fresh medium and continuing the incubation. The chelators were removed after 5 h and 8 h and the incubation was continued for 10 h and 7 h respectively. The effects of chelators at later growth stages...
were examined by adding them after 5 h (when conidia start to swell) or 8 h (when conidia start to germinate) had passed and the incubation was continued for 10 h and 7 h respectively (34). For growth under hypoxic conditions plates were placed in an AnaeroJar (AG0025, Oxoid) according to the manufacturer’s instructions in order to generate an environment with < 0.1% O₂.

The interactions between caspofungin and the chelators were ascertained by determining the fractional inhibitory concentration index (FICI) using a checkerboard method (39). Caspofungin was selected as an example of an established antifungal drug with a mode of action blocking the synthesis of β(1,3)-d-glucan of the fungal cell wall, that differs from that of the chelators acting on zinc metabolism tested in this study. The concentrations used in this assay were closer to one another than those in a two-fold dilution series in order to obtain greater resolution. MIC-2 (50% inhibition in luminescence) was employed as an end point, as caspofungin is cytostatic rather than cytotoxic (40). The FICI was defined as FICI = (Ac/Aa) + (Bc/Ba), where Ac and Bc are the MIC-2 of the chelator and caspofungin in combination, Aa is the MIC-2 of the chelator and Ba is the MIC-2 of caspofungin. Interactions were classified as synergistic (FICI ≤ 0.5), indifferent (FICI > 0.5 but ≤ 4), or antagonistic (FICI > 4).

*Murine infection and in vivo bioluminescence imaging using an IVIS 100 system.*

We used our model of invasive pulmonary aspergillosis (34). Male BALB/cJ mice (23 to 28 g, 8 weeks old) supplied by the breeding center R. Janvier (Le Genest Saint-Isle, France) were used in these experiments. 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 CETEA (Comité d’éthique en experimentation animale, Project license number 2013-0020). Mice were weighed daily to monitor changes in body weight. Four and one day before infection each mouse received an immunosuppressive regimen by 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 were inoculated intranasally with a dose of 5x10^4 conidia in 25 μl of PBS-0.01 %Tween. Following infection, the chelators, caspofungin or placebo were administered by i.p. injection at the indicated concentrations in a final volume of 100 μl. The placebo consisted of 10% DMSO in saline solution. The initial concentrations tested for the chelators were based on existing information on murine toxicity of the compounds. The LD50 for intraperitoneal injection of phenanthroline in mice is 75 mg/kg (41). Intraperitoneal injection of ≤ 10 mg/kg TPEN was well tolerated, while ≥ 30 mg/kg resulted in death (42). We addressed this problem by using a lower dose of each chelator. The experiments were repeated in order to confirm reproducibility. Combining all the experiments resulted in the control group contained 55 mice, the 5 mg/kg/day TPEN group 25 mice, the 10 mg/kg/day phenanthroline group 30 mice the 10 mg/kg/day caspofungin group 15 mice, the 5 mg/kg/day caspofungin and 5 mg/kg/day TPEN group 25 mice. 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 (43). Experiments lasted for
14 days post-infection, including 10 days of daily treatment followed by 4 days without treatment to monitor weight recovery.

**Inflammatory mediator quantification.**

Dead mouse lungs were disrupted in saline using the Retsch Mixer Mill 301 homogenizer. ELISA was used to determine IL-6 and CXCL1 concentrations in lung supernatants as specified by the manufacturer (DuoSet; R&D Systems).

**Statistical analyses.**

For the *in vitro* tests, the luminescence values of the different cultures in the presence of chelators and/or metal ions were compared to those of the control cultures using unpaired t-tests with Welch's correction. Levels of significance for hyphal lengths were calculated using the Mann-Whitney test. For the *in vivo* tests, survival rates were performed by creating Kaplan-Meier plots and then performing log rank tests. All results are expressed as means ± standard errors of the mean (SEM), and comparisons for survival studies were considered significant if the p value was < 0.05. Comparisons of body weights and luminescence within the different groups of mice were performed using the Mann-Whitney test. All tests were performed using GraphPad Prism 6 software. All the MIC-2 and MIC-0 values reported were statistically significant and the p-values indicate the level of significance compared to the positive controls.

**Results**
The chelators clioquinol, phenanthroline and TPEN were highly effective at inhibiting *A. fumigatus* growth. DEDTC, DTPA, EDDA and EDTA all had MIC-0 and MIC-2 values equal to or greater than 112.5 mg/L, while clioquinol, phenanthroline and TPEN all had MIC-0 and MIC-2 values equal to or less than 6 mg/L (Table 1). Clioquinol, phenanthroline and TPEN were thus selected for further experiments, as they were the most effective inhibitors among the compounds examined in this study. The MIC or MEC according to the EUCAST microdilution method after 48 h incubation was 0.125 mg/L (MIC) for clioquinol, 4 mg/L (MIC) for phenanthroline and 8 mg/L (MIC) and 0.125 mg/L (MEC) for TPEN. Both MIC and MEC are presented for TPEN because it was able to cause highly stunted growth at levels that were much lower than those causing complete cessation of growth. These values differ from those on Table 1 due to the difference in medium composition and method.

**Zinc neutralized both phenanthroline and TPEN.** To test the capacity of different metals to overcome the inhibitory effects of clioquinol, phenanthroline and TPEN on fungal growth, conidia were inoculated in media containing increasing amounts of these chelators and different salts of metallic ions at a concentration of 1 mM. Fe^{2+} only had a strong effect on clioquinol and little effect on phenanthroline and TPEN, suggesting that the latter two chelators did not bind it (Fig. 1). Mg^{2+} had no significant effect on any of the chelators tested, indicating that none of them bound it. Mn^{2+} had a moderate effect only on TPEN. Zn^{2+} completely abolished the inhibition of phenanthroline and TPEN but had little effect on clioquinol, indicating that
the latter did not sequester Zn in the tested conditions tested. In summary, clioquinol is
strongly inhibited by Fe, phenanthroline is strongly inhibited by Zn and TPEN is strongly
inhibited by Zn and moderately inhibited by Mn.

Chelators had an inhibitory effect that was not growth phase-dependent.
The level of inhibition was time-sensitive, as it was reduced if the chelators were left for
a shorter period of time (5 h rather than 8 h) in the presence of conidia before being
removed (Fig. 2). Clioquinol was faster-acting than phenanthroline, which in turn was
faster-acting than TPEN. The high level of inhibition after chelator removal via washing
and replacement of the medium suggests that they were fungicidal after 8h incubation.
Note however that this result relies on fungal inability to resume growth rather than on
the determination of a MIC and is therefore only indicative. All of the chelators were able
to inhibit growth when added after the conidia had swollen (after 5 h of incubation) or
germinated (after 8 h of incubation).

Phenanthroline and TPEN were unaffected by hypoxia, while mammalian sera
caus ed a reduction in clioquinol and TPEN efficacy.
Chelator efficacy was tested under hypoxic conditions and in the presence of mammalian
sera in order to simulate the conditions within a host. After 15 hours of incubation *A.
fumigatus* grown under hypoxic conditions without chelators had luminescence ranging
from 3% to 5% of those for the fungus grown under normoxic conditions (Fig. S1a). Both
the MIC-2 and MIC-0 of clioquinol underwent a significant reduction (p = 0.0001) under
hypoxia when compared to normoxia (Fig. S1b). No significant differences were
observed in the MIC values for the other chelators. These findings indicate that hypoxia did not influence significantly the efficacy of phenanthroline and TPEN whereas it appeared to decrease that of clioquinol.

*A. fumigatus* grown in RPMI supplemented with 10% human serum showed luminescence that was one fourth that of FCS-supplemented RPMI. Rat and mouse serum had one tenth and one twentieth as much respectively, while rabbit serum and saline, used as a negative control, had similar levels of luminescence to FCS-supplemented RPMI (Fig. S2a). Clioquinol was most effective in medium containing saline, followed by human, mouse rabbit and rat serum, all of which had similar effects, and was least effective in medium containing fetal calf serum (p < 0.0001) (Fig. S2b-d). Phenanthroline was almost equally effective with all sera tested and with saline. TPEN was most effective in medium containing saline (p < 0.0001), followed by human and mouse sera (p < 0.0001) and was least effective in the presence of fetal calf, rabbit and rat sera.

Caspofungin had an indifferent effect with both phenanthroline and TPEN *in vitro*. Caspofungin had a MIC-2 of 0.069 mg/L but no MIC-0, as it was fungistatic rather than fungicidal to *Aspergillus* and could not cause a 90% reduction in growth (40). Caspofungin and phenanthroline in combination had a MIC-2 of 0.046 mg/L and 0.5 mg/L respectively, while separately these values respectively were 0.069 mg/L and 1 mg/L. Their FICI (fractional inhibitory concentration index) was 1.17. Caspofungin and TPEN in combination had a MIC-2 of 0.023 mg/L and 2 mg/L respectively, while separately these values respectively were 0.069 mg/L and 3 mg/L. Their FICI was
Phenanthroline and TPEN were effective in treating IPA in immunosuppressed mice infected with *A. fumigatus*. The three most successful chelators in vitro were tested in vivo to determine their effectiveness when used to treat an infected animal model. Mice in the 10% DMSO control group demonstrated a 16% survival rate, with 9 out of 55 mice still alive at the end of the experiment. Clioquinol was tested on infected mice at concentrations of 30 and 15 mg/kg/day. These treatments either decreased or did not significantly alter mouse survival and clioquinol was abandoned (Fig. S3). TPEN was tested at concentrations of 10, 7.5 and 5 mg/kg/day, while phenanthroline at concentrations of 30, 15 and 10 mg/kg/day. The best effects were observed at concentrations of 5 mg/kg/day for TPEN and 10 mg/kg/day for phenanthroline. Mice treated with 5 mg/kg/day TPEN demonstrated 60% survival, with 15 out of 25 mice alive at the end of the experiment, while those treated with 10 mg/kg/day phenanthroline had 47% survival with 14 out of 30 mice alive at the end of the experiment (Fig. 4a). Both the phenanthroline and TPEN groups showed weight loss on days 3 and 4 that was significantly lower than the control Fig. 4b). The TPEN group demonstrated significantly less luminescence than the control on days 3 and 5 while the phenanthroline group had significantly less luminescence on day 5 (Fig. 4c-d). In summary, both TPEN and phenanthroline significantly increased survival of mice suffering from pulmonary aspergillosis.
TPEN and caspofungin had an indifferent effect in vivo. To analyze the effect of TPEN plus caspofungin in vivo we first determined the optimal concentration of caspofungin for combination treatment and found that 1 mg/kg/day (15 mice) resulted in 100% survival, 0.75 mg/kg/day (10 mice) in 90% survival, 0.5 mg/kg/day (15 mice) in 53% survival and 0.25 mg/kg/day (5 mice) in 20% survival. All but the lowest caspofungin concentration had significantly higher survival compared to the control (Fig. S4). A combination of 0.5 mg/kg/day caspofungin and 5 mg/kg/day TPEN gave 88% survival, with 22 out of 25 mice alive at the end of the experiment. In addition, the survival outcome of the combined treatment (caspofungin plus TPEN) turned out to be significantly higher compared to that using either caspofungin or TPEN alone (Fig. 5a). The combination therapy also significantly reduced the weight loss as compared to the control group on days 2 to 5. In addition, it significantly reduced the weight loss compared to caspofungin monotherapy (Fig. 5b). Finally, the combination therapy and the TPEN treatment allowed a very high reduction in the levels of luminescence compared to the control and caspofungin (Fig. 5c). In summary, TPEN and caspofungin combination significantly improved mouse survival compared to using either compound individually.

Infection causes exacerbated inflammation patterns in the lungs.

The effects of infection and treatment on the levels of two inflammatory cytokines (IL-6 and CXCL1) within the lungs were examined using ELISA either on mice that died between days 4 and 5 (Fig. 6a) or mice that survived the duration of the experiment and were euthanized on day 14 (Fig. 6b). All the mice that died on days 4 and 5 demonstrated...
high levels of inflammation with levels of IL-6 between 2400 and 5000 pg/100mg and

CXCL1 between 5000 and 10000 pg/100mg. There was no significant difference between
treated and untreated mice. The surviving mice all had relatively low levels of cytokines,
though the mice treated with chelators or chelator and caspofungin combination had
cytokine levels significantly higher than the uninfected mice that received TPEN.

Discussion

We analyzed the effect of several Zn-targeting chelators on fungal growth in vitro prior to
testing their efficacy in vivo using a murine model of IPA. Our in vitro assays
demonstrated that clioquinol, phenanthroline and TPEN were the most effective
independently of fungal growth stage and they were selected for use in vivo. They all
produced complete inhibition of fungal growth at low concentrations and have been
previously used in animal models (18, 30, 33). In our study, we have shown that phenanthroline and TPEN bound specifically to zinc
and not to iron. Their effects are neutralized by zinc. It has been shown that
phenanthroline is able to prevent growth of the filamentous fungus Phialophora
verrucosa by inhibiting a zinc-dependent metallopeptidase and interfering with fungal
morphogenesis (44). In addition previous research demonstrated that TPEN decreases
available zinc but not iron in vitro (45, 46). Moreover, the cytotoxic effect of TPEN is
thought to be due to the chelation of intracellular zinc, which interferes with the
functioning of essential metalloproteins (47). This is supported by the fact that TPEN
strongly promotes the expression of zinc transporter proteins in the fungal pathogen
Cryptococcus gattii (48) and the yeast Paracoccidioides brasiliensis (49). Furthermore, an Acinetobacter baumannii strain with a knocked out zinc transporter is more sensitive to TPEN than the wild type (50).

Testing whether the efficacy of the chelators is affected by hypoxia was important to perform in order to more closely simulate their effects in vivo. It has been shown that inflammation of the lung causes extensive tissue destruction that leads to hypoxic conditions (51, 52). In addition, hypoxia can alter the efficacy of antifungal drugs on Aspergillus, causing an increase in the inhibition by amphotericin B, micafungin and anidulafungin, a slight decrease in the efficacy of itraconazole and no effect on voriconazole (53). The effectiveness of azoles is reduced under hypoxic conditions due to the reduction in ergosterol biosynthesis, which is one of the pathways more dependent on pO2. In our study, we found that hypoxia has a strong inhibitory effect on A. fumigatus growth as previously reported (54). Under hypoxic conditions, the effectiveness of phenanthroline and TPEN remained unchanged, which further increases their potential clinical value as their activity is unaffected by the low oxygen levels found in hypoxic lung tissue.

Blood serum can affect the growth of different Aspergillus strains in varying degrees as well as the efficacy of different antifungal compounds either positively or negatively (55); this can vary depending on which species the serum originates from (56). Additionally, there is a correlation between the efficacy of certain antifungals in the presence of serum in vitro and in vivo (57). For these reasons we decided to compare the effects of the presence or absence of human and other animal sera on A. fumigatus and zinc chelators, as this may provide information regarding their in vivo efficacy. The
inhibition of *A. fumigatus* by human serum agrees with previous findings (55) and is due
to the fact it contains fungal inhibitors (58). The effectiveness of phenanthroline was not
affected by any of the sera, however all tested sera reduced the effectiveness of both
clioquinol and TPEN; an effect also observed on other antifungal compounds such as
amphotericin B and echinocandins (55). This may be due to binding by albumin or other
plasma proteins (59, 60), though there are likely to be other unknown factors affecting
their efficacy as well (57).

Though the serum tests suggested that phenanthroline might be more effective *in vivo*
because it was not inhibited by the components of the serum, TPEN proved to be slightly
more effective in treating aspergillosis. It may be that phenanthroline is more readily
metabolized by the host and/or that it has a greater difficulty in reaching the lungs.

Our findings demonstrate that zinc chelators can be used to improve survival, to decrease
the severity of disease symptoms and to decrease the fungal burden of the host.

Use of a chelator to sequester a trace metal required for growth by a fungal pathogen has
previously been employed against iron. Deferiprone and deferasirox have been
successfully used to treat mucormycosis and aspergillosis in mice (61) (62) (63, 64) and
human patients (65-67). Collectively these findings and our data indicate that zinc and
iron chelators do not interfere with other key metabolic processes of the host and can be a
promising treatment option.

Treatment using combinations of different antifungal drugs has been suggested to address
the development of resistance among pathogens, as well as to reduce side-effects due to
drug toxicity to the patient and to achieve antimicrobial synergy (68). Combination
treatments are widely used for treating aspergillosis as well as other infections (69). For
instance, iron chelators have been used in combination therapy against fungal infections both in laboratory animals (62-64, 70) and in humans (65-67). Combination treatment is thought to improve survival in both patients and animal models (39, 71). Different drug combinations may be synergistic, indifferent or antagonistic against *Aspergillus in vitro* (72). The echinocandin antifungal caspofungin (73) was selected as a combination drug because it has favorable outcomes for treating aspergillosis in clinical trials and has been recommended as a treatment option for the disease either as a monotherapy or in combination with other drugs (74). Our *in vitro* results demonstrated that both phenanthrolin and TPEN had indifferent effects when used in combination with caspofungin. This finding agrees with previous findings using EDTA (12) or iron chelators in combination to polyenes (75). The combination treatment of caspofungin and TPEN demonstrated an indifferent effect and significantly improved survival compared to both TPEN and caspofungin monotherapy. Again this result agrees with the finding that an iron chelator in combination with other antifungals was able to improve survival of mice suffering from aspergillosis (64).

The elevated cytokine levels in the lungs of mice that succumbed to pulmonary aspergillosis indicate that these mice died from exacerbated inflammation. This has been observed in chronic granulomatous disease, where patients can succumb to *Aspergillus* infections from hyperinflammation (76). In our study, the elevated cytokine levels in surviving mice treated with the chelators suggesting that the mice had not yet fully returned to normal 14 days post-infection even though no *Aspergillus* growth was visible in the lungs using luminescence measurements.
In summary, based on both in vitro and in vivo assays we conclude that the zinc chelators phenanthroline and TPEN are able to function effectively as antifungal drugs for the treatment of pulmonary aspergillosis in mice either as a monotherapy or as part of a combination therapy. For this reason we are currently undertaking a large scale in vitro screen of around 60,000 natural and synthetic small molecules in order to identify novel compounds that might interfere with zinc metabolism and may lead to additional treatment options for invasive pulmonary aspergillosis.

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References


Figure Legends:

Fig. 1: Percent inhibition of A. fumigatus grown for 15 h in the presence of 1 mM of FeSO4, MgSO4, MnSO4, ZnSO4 or ZnCl2 in addition to (a) clioquinol, (b) phenanthroline and (c) TPEN.

Fig. 2: Percent inhibition of A. fumigatus grown in the presence of (a) clioquinol, (b) phenanthroline and (c) TPEN with the chelators either added after 5 or 8 hours after the initiation of incubation or removed after an initial incubation for 5 or 8 hours in their presence, with a total incubation time of 15 h.

Fig. 3: Heat maps of percent inhibition of varying concentrations of the antifungal caspofungin in combination with varying concentrations of the chelator (a) phenanthroline or (b) TPEN.
Fig. 4: In vivo comparison of treatments on mouse survival using 10% DMSO placebo, 5 mg/kg/day TPEN and 10 mg/kg/day phenanthroline: (a) Survival curves (phenanthroline vs control: p = 0.0023, TPEN vs control: p < 0.0001), (b) weight averages (Day 3: phenanthroline vs control: p = 0.0116, TPEN vs control: p = 0.0489. Day 4: phenanthroline vs control: p = 0.0333, TPEN vs control: p = 0.0024), (c) luminescence averages (Day 3: TPEN vs control: p = 0.0122, Day 5: phenanthroline vs control: p = 0.0169, TPEN vs control: p = 0.0140). (d) Examples showing luminescence of mice treated with 5 mg/kg/day TPEN, treated with 10 mg/kg/day phenanthroline and of a 10% DMSO placebo group. As indicated in the scale bar (2.5x10E5 – 4x10E5 total photons/sec), mice with low levels of luminescence (blue) did not develop an infection and survived. Mice with high levels of luminescence (red) developed pulmonary aspergillosis and succumbed to it.

Fig. 5: In vivo comparison of treatments on mouse survival using 10% DMSO placebo, 5 mg/kg/day TPEN, 5 mg/kg/day Caspofungin and a Combination of TPEN and Caspofungin (a) Survival curves (Combination vs Control: p < 0.0001, Combination vs Caspofungin: p = 0.0084, Combination vs TPEN: p = 0.0223), (b) weight averages (Day 2: Combination vs Placebo: 0.0057. Day 3: Combination vs Placebo: 0.0057, TPEN vs Placebo: 0.0116, Combination vs Caspofungin: 0.0119. Day 4: Combination vs Placebo: < 0.0001, TPEN vs Placebo: 0.0333, Combination vs Caspofungin: 0.0439. Day 5: Combination vs Placebo: 0.0048, Combination vs Caspofungin: 0.0218) (c) luminescence (Day 3: Combination vs Placebo: 0.0070, TPEN vs Placebo: 0.0122, Caspofungin vs Placebo: 0.0130. Day 5: Combination vs Placebo: 0.0067, TPEN vs Placebo: 0.0140)
averages of the 10% DMSO placebo group the 5 mg/kg/day caspofungin, 5 mg/kg/day caspofungin and 5 mg/kg/day TPEN combination and 5 mg/kg/day TPEN treated groups.

Fig. 6: Levels of the inflammatory cytokines IL-6 and CXCL1 in (a) untreated and treated mice that died from pulmonary aspergillosis infection and (b) treated mice that survived the infection. The deceased mouse groups included 8 placebo, 5 caspofungin (0.5 mg/kg/day), 5 phenanthroline (10 mg/kg/day) and 1 TPEN (5 mg/kg/day) mouse. The survivor group included 4 uninfected mice that received TPEN (5 mg/kg/day), 6 caspofungin (1 mg/kg/day), 7 phenanthroline (10 mg/kg/day), 7 combination (5 mg/kg/day TPEN and 0.5 mg/kg/day caspofungin) and 10 TPEN (5 mg/kg/day) mice. No significant differences were observed among the deceased mice. Phenanthroline vs uninfected: 0.0468 for IL-6 and 0.0169 for CXCL1, Combination vs uninfected: 0.0424 for IL-6 and 0.0338 for CXCL1 and TPEN vs uninfected: 0.0204 for IL-6 and 0.0022 for CXCL1.
Table 1: Inhibition of luminescence of *A. fumigatus* grown for 15 h in the presence of different concentrations of selected chelators.

<table>
<thead>
<tr>
<th>Chelator</th>
<th>MIC-0 (mg/L)</th>
<th>MIC-2 (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clioquinol</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>DEDTC</td>
<td>900</td>
<td>337.5</td>
</tr>
<tr>
<td>DTPA</td>
<td>800</td>
<td>300</td>
</tr>
<tr>
<td>EDDA</td>
<td>&gt; 700</td>
<td>&gt; 700</td>
</tr>
<tr>
<td>EDTA</td>
<td>&gt; 1500</td>
<td>750</td>
</tr>
<tr>
<td>Phenanthroline</td>
<td>2</td>
<td>0.5</td>
</tr>
<tr>
<td>TPEN</td>
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<td>3</td>
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</tbody>
</table>
Figure 2
### Figure 3

(a) **Phenanthroline**

<table>
<thead>
<tr>
<th>Caspofungin</th>
<th>2 mg/L</th>
<th>1.5 mg/L</th>
<th>1 mg/L</th>
<th>0.5 mg/L</th>
<th>0.2 mg/L</th>
<th>0 mg/L</th>
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<tbody>
<tr>
<td>0.090 mg/L</td>
<td>98.51</td>
<td>97.02</td>
<td>90.52</td>
<td>76.12</td>
<td>65.72</td>
<td>57.68</td>
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<tr>
<td>0.069 mg/L</td>
<td>98.40</td>
<td>97.21</td>
<td>90.24</td>
<td>77.12</td>
<td>61.27</td>
<td>57.83</td>
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<tr>
<td>0.046 mg/L</td>
<td>98.60</td>
<td>97.35</td>
<td>90.06</td>
<td>72.63</td>
<td>54.67</td>
<td>46.89</td>
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<td>96.72</td>
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<td>49.78</td>
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<td>18.73</td>
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<tr>
<td>0.009 mg/L</td>
<td>98.65</td>
<td>96.73</td>
<td>67.37</td>
<td>17.21</td>
<td>6.63</td>
<td>-0.09</td>
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<tr>
<td>0 µg/L</td>
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<td>96.60</td>
<td>73.35</td>
<td>14.28</td>
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(b) **TPEN**

<table>
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<tr>
<th>Caspofungin</th>
<th>4 mg/L</th>
<th>3 mg/L</th>
<th>2 mg/L</th>
<th>1 mg/L</th>
<th>0.4 mg/L</th>
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<td>80.70</td>
<td>58.53</td>
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<td>59.71</td>
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<td>0.069 mg/L</td>
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<td>90.51</td>
<td>53.15</td>
<td>57.07</td>
<td>56.99</td>
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<tr>
<td>0.046 mg/L</td>
<td>99.10</td>
<td>98.65</td>
<td>73.10</td>
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<td>10.44</td>
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<tr>
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<td>98.57</td>
<td>26.00</td>
<td>2.85</td>
<td>6.10</td>
<td>3.33</td>
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<tr>
<td>0 mg/L</td>
<td>99.09</td>
<td>98.43</td>
<td>19.48</td>
<td>3.53</td>
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<td>0.00</td>
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