Synthetic Derivatives of Ciclopirox are Effective Inhibitors of Cryptococcus neoformans

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Abstract: Opportunistic fungal infections caused by Cryptococcus neoformans are a significant source of mortality in immunocompromised patients. They are challenging to treat because of a limited number of antifungal drugs, and novel and more effective anticytoccocal therapies are needed. Ciclopirox olamine, a N-hydroxypyridone, has been in use as an approved therapeutic agent for the treatment of topical fungal infections for more than two decades. It is a fungicide, with broad activity across multiple fungal species. We synthesized 10 N-hydroxypyridone derivatives to develop an initial structure–activity understanding relative to efficacy as a starting point for the development of systemic antifungals. We screened the derivatives for antifungal activity against C. neoformans and Cryptococcus gattii and counter-screened for specificity in Candida albicans and two Malassezia species. Eight of the ten show inhibition at 1–3 μM concentration (0.17–0.42 μg per mL) in both Cryptococcus species and in C. albicans, but poor activity in the Malassezia species. In C. neoformans, the N-hydroxypyridones are fungicides, are not antagonistic with either fluconazole or amphotericin B, and are synergistic with multiple inhibitors of the mitochondrial electron transport chain. They appear to function primarily by chelating iron within the active site of iron-dependent enzymes. This preliminary structure–activity relationship points to the need for a lipophilic functional group at position six of the N-hydroxypyridine ring and identifies positions four and six as sites where further substitution may be tolerated. These molecules provide a clear starting point for future optimization for efficacy and target identification.

INTRODUCTION

Cryptococcus neoformans is a fungal pathogen of immunocompromised people that causes up to 1 million infections each year among HIV-positive patients globally, resulting in up to 250,000 deaths annually.1 Cryptococcal infections are the third leading cause of infections among solid organ transplant patients and transplant patients remain susceptible to C. neoformans infections for several years because of its presence in the environment.2−4 C. neoformans infections can be treated with amphotericin B (AMB) and fluconazole (FLC), but the treatment course is long and has significant toxicity. The mortality rate for cryptococcal infections remains 15−30% even in the context of antiviral treatments for HIV.5−7 Thus, there is a significant need for safer and more effective drugs for treating cryptococcal infections.

Ciclopirox olamine (CPO) (Table 1), a N-hydroxypyridone (HPO), has been in use as an approved therapeutic agent for the treatment of topical fungal infections since 1998. It is a fungicide with broad activity across multiple fungal species.8 It has been assessed for efficacy in treating systemic fungal infections, viral infections, and some cancers.9 In mammalian systems, CPO has been shown to inhibit cell proliferation and induce cell death by inhibition of ribonucleotide reductase, an iron-dependent enzyme.10 More recently, CPO and derivatives have been tested for applying as potential anti-ischemic stroke agents.11 Two recent studies support the potential of CPO for a systemic treatment. A recent phase I clinical study of oral ciclopirox found that oral dosing of CPO was well tolerated, although higher and more frequent doses resulted in gastrointestinal toxicity.12 The use of CPO for the treatment of congenital erythropoietic porphyria was evaluated in a mouse model.13 These studies suggest that CPO may be of therapeutic benefit but will need to be reformulated to improve half-life and toxicity.

Although it was approved as a topical antifungal agent more than two decades, the mechanism of action in fungi is not completely understood. CPO is able to chelate polyvalent...
metal cations, particularly Fe in both oxidation states. This results in inhibition of metal-dependent enzymes, notably cytochromes, catalases, and peroxidases, which can disrupt mitochondrial function, energy production, and transport across membranes. Studies of different ascomycete species suggest that inhibition of iron permeases and transporters contributes to their toxicity in fungi.\textsuperscript{14,15} CPO is a fungicide and no resistance has been reported despite more than three decades of clinical use. Attempts to generate resistant \textit{Candida albicans} strains in the laboratory have also been unsuccessful despite six-month treatment of cells with sublethal levels of CPO.\textsuperscript{15} A preclinical study examined the efficacy of CPO against 16 clinical strains of \textit{C. neoformans} and measured fungicidal minimal inhibitory concentrations (MICs) between 0.5 and 4 \(\mu\)g/mL (2–16 \(\mu\)M).\textsuperscript{16} We believe that this chemical scaffold is worth exploring for developing potentially new and more effective inhibitors of \textit{C. neoformans}.

We synthesized 10 HPO derivatives (Table 1) of CPO to explore the preliminary structure–activity relationships (SAR) with respect to their efficacy as an antifungal agent and toxicity in mammalian cell culture. We also explored possible mechanisms of action of three HPOs in \textit{C. neoformans}, a basidiomycete yeast. \textit{C. neoformans} is capable of surviving and replicated within human macrophages, a highly oxidative environment. We hypothesize that the toxicity of CPO and derivatives will be primarily driven by their ability to inhibit iron- or other multivalent metal-dependent enzymes essential for metabolic activities.

\section*{RESULTS AND DISCUSSION}

\textbf{Chemical Synthesis.} Analogs of CPO are not readily available. In an effort to develop SARs, analogs were prepared through two primary synthetic routes. Synthesis via \(\alpha\)-pyrones is depicted in Schemes 1 and 2 and via 2-methoxypyridines in Schemes 3 and 4.

The desmethyl analog (9) of CPO was synthesized as shown in Scheme 1 by improvising the procedure reported by Dong et al.\textsuperscript{17} Cyclohexylcarboxylic acid 1 was activated with thionyl chloride and treated with benzotriazole to generate acyl benzotriazole 3.\textsuperscript{18} Generation of the lithium enolate of 4 with

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|c|c|c|}
\hline
Structure & Compound ID & \(\text{ClogP}\) & \(\text{C. neo}^{b}\)MIC\(_{50}\) \(\mu\)M (\(\mu\)g/mL) & \(\text{C. neo}^{c}\)MIC\(_{50}\) \(\mu\)M & \(\text{C. gattii}^{d}\)MIC\(_{50}\) \(\mu\)M & \(\text{C. albicans}^{c}\)MIC\(_{50}\) \(\mu\)M & SI \(^{e}\) \\
\hline

\includegraphics[width=0.3\textwidth]{structure1} & Ciclopirox & 2.2 & 0.87 (0.18) & 27.7 & 32 & 1.7 & 2.3 & 1.9 \\
\hline

\includegraphics[width=0.3\textwidth]{structure2} & *SLU-2350 (9) & 2.0 & 0.87 (0.17) & 16.5 & 19 & 1.2 & 1.7 & 1.9 \\
\hline

\includegraphics[width=0.3\textwidth]{structure3} & *SLU-2324 (14a) & 1.6 & 1.2 (0.24) & 18 & 15 & 2.3 & 2.3 & 3.9 \\
\hline

\includegraphics[width=0.3\textwidth]{structure4} & SLU-2321 (14b) & 2.6 & 1.5 (0.33) & 7.8 & 5 & 1.7 & 1.7 & 1.5 \\
\hline

\includegraphics[width=0.3\textwidth]{structure5} & *SLU-2318 (14c) & 2.1 & 1.2 (0.26) & 9.6 & 8 & 2.3 & 1.7 & 1.2 \\
\hline

\includegraphics[width=0.3\textwidth]{structure6} & SLU-2684 (16a) & 2.0 & 1.1 (0.27) & 11.3 & 10 & 3.2 & 1.5 & 2.7 \\
\hline

\includegraphics[width=0.3\textwidth]{structure7} & SLU-2681 (16b) & 2.0 & 1.1 (0.27) & 15.4 & 14 & 2.3 & 2.3 & 2.3 \\
\hline

\includegraphics[width=0.3\textwidth]{structure8} & SLU-2709 (16c) & 2.0 & 1.2 (0.29) & 15.1 & 13 & 2.3 & 2.3 & 2.3 \\
\hline

\includegraphics[width=0.3\textwidth]{structure9} & SLU-2707 (16d) & 2.7 & 1.7 (0.42) & 10.2 & 6 & 3 & 3 & 1.7 \\
\hline

\includegraphics[width=0.3\textwidth]{structure10} & SLU-2237 (20) & 1.8 & 12 (2.4) & 80 & 6.7 & 9 & 9 & 38 \\
\hline

\includegraphics[width=0.3\textwidth]{structure11} & SLU-2239 (25) & 1.5 & 31 (5.8) & 75 & 1.5 & 14 & 9 & 50 \\
\hline
\end{tabular}
\caption{Structures and Data for HPO Derivatives}
\end{table}
in situ-generated LDA gave ketone 5. Cyclization in refluxing toluene gave 4-hydroxy-α-β-pyrene 6. Treatment with phosphorous tribromide gave bromide 7, which was subsequently reduced with zinc to give α-β-pyrene 8. Final conversion of 8 to HPO 9 was accomplished by heating with hydroxylamine hydrochloride and imidazole with a poor yield over the three steps. After we had completed this sequence, Hu and co-workers reported a complementary approach to the synthesis of 9.11

The synthesis of 4-methyl-6-substituted HPO R⁵ analogs 14a–c and 16a–d was achieved as illustrated in Scheme 2 by adaptation of the procedure reported by Liu et al.19 Reaction of commercially available ethyl 3-methylbut-2-enoate 10 with its corresponding freshly prepared acyl chlorides 11a–d in the presence of AlCl₃ furnished a differently substituted stereoisomeric mixture of esters. Cyclization of crude α,β-unsaturated esters 12a–d by heating in a mixture of acetic acid and sulfuric acid generated pyron-2-one intermediates 13a–d. Reaction of intermediates 13a–c with hydroxylamine hydrochloride and imidazole under neat conditions at 100 °C afforded the target compounds 14a–c.

6-Chloromethyl substituted pyron-2-one intermediate 13d was further derivatized by using different aryl boronic acids under mild Suzuki coupling conditions to provide compounds 15a–d. The intermediates 15a–d were converted to corresponding final targets 16a–d following a similar procedure utilized for the synthesis of 14a–c.

R⁵ derivative 22 was synthesized as shown in Scheme 3. Bromine–lithium exchange of compound 17 with n-BuLi in tetrahydrofuran (THF) at −78 °C followed by a reaction of resulting 5-lithio species with benzaldehyde 18 afforded the alcohol 19. The conversion of the benzyl alcohol 19 to benzyl pyridine 20 was achieved by reduction with Pd/C in methanol under a hydrogen atmosphere in the presence of a catalytic amount of trifluoroacetyl (TFA). The oxidation of the pyridine ring in compound 20 was achieved by heating in chloroform at

Scheme 1. Synthesis of Desmethyl Analog 9

Scheme 2. Synthesis of Cyclohexyl Replacement Analogs 12a–c

Scheme 3. Synthesis of R⁵ Benzyl Analog 22

Scheme 4. Synthesis of R⁴ Phenyl Analog 25

Reagents and conditions: (a) AlCl₃, DCM; (b) AcOH, H₂SO₄, 100 °C, 18–54% for 13a,c,d; 24% over three steps for 13b; (c) hydroxylamine hydrochloride, imidazole, 100 °C, 22–69% for 14b–c and 16a–d; 0.5% over four steps for 14a; and (d) aryloboronic acid, Pd(dppf)Cl₂, K₂CO₃, 1,4-dioxane, 100 °C, 43–77%.

Reagents and conditions: (a) thionyl chloride, DCM, 5 °C to room temp; (b) n-BuLi, DIPA, THF, −78 °C to room temp, 28%; (c) toluene, reflux, 92%; (d) PBr₃, DMF, −15 to 60 °C; (e) zinc, HCl, EtOH; and (f) hydroxylamine hydrochloride, imidazole, 100 °C, 1.3% over three steps.

Reagents and conditions: (a) n-BuLi, THF, −78 °C, 72%; (b) H₂, Pd/C, TFA, MeOH; (c) m-CPBA, CHCl₃, 50 °C, 55%; and (d) (i) AcCl, 50 °C; (ii) K₂CO₃, MeOH, 19%.

Reagents and conditions: (a) PdCl₂(PPh₃)₂, K₂CO₃, DME, H₂O, 80 °C, 90%; (b) m-CPBA, CHCl₃, 50 °C, 63%; and (c) (i) AcCl, 50 °C, (ii) K₂CO₃, MeOH, 5%.
50 °C as described by Liu and Zheng.\textsuperscript{19,20} The pyridine N-oxide compound 21 was converted to HPO compound 22 by reacting with acetyl chloride at 50 °C followed by stirring the reaction in methanol at room temperature.

R\textsuperscript{4} analog 27 was achieved by using Suzuki coupling as the key step as depicted in Scheme 4. Reaction of 4-bromo-2-methoxypyridine 23 with phenyl boronic acid 24 in the presence of PdCl\textsubscript{2}(PPh\textsubscript{3})\textsubscript{2} and K\textsubscript{2}CO\textsubscript{3} in dimethoxyethane and water gave the compound 25. Compound 25 was converted to the desired target 27 following a similar procedure used in the conversion of 21 to 22 reported by Zheng et al.\textsuperscript{20}

**HPOs Inhibit C. neoformans, Cryptococcus gattii, and C. albicans.** All 10 HPO derivatives of CPO were tested for efficacy against the laboratory and a clinical strain of C. neoformans and a clinical strain C. gattii using a microdilution assay described previously.\textsuperscript{21} All 10 HPOs were counter-screened in C. albicans and a subset was tested for efficacy in Malassezia furfur and Malassezia pachydermatis. The MIC\textsubscript{50} value for CPO against the laboratory C. neoformans strain (KN99) was 0.87 μM (0.18 μg/mL) in this assay (Table 1). We are reporting MIC\textsubscript{50} values in micromolar to facilitate comparison across the chemical derivatives. In C. neoformans, eight of the 10 derivatives had MIC\textsubscript{50} values <2 μM (Table 1). Cytotoxicity of the compounds after 72 h of exposure was determined using an MTS toxicity assay in Huh7, a human liver hepatoma cell line. The CC\textsubscript{50} value was determined as the concentration of the compound needed to inhibit 50% of the cells relative to a vehicle control. The ratio of CC\textsubscript{50}/MIC\textsubscript{50} is the selectivity index (SI). The SI values for this set of compounds ranged from 1.5 to 19 compared to a SI of 39 for the selectivity index (SI). The SI values for this set of compounds ranged from 1.5 to 19 compared to a SI of 39 for CPO under the same conditions. All 10 HPOs were tested for inhibition of FLC-resistant clinical isolates of C. neoformans (DUMC 158.03) and C. gattii (RSA-MW-3615).\textsuperscript{22} The MIC\textsubscript{50} values ranged from 1.7 to 14 μM in the C. neoformans isolate and from 1.7 to 9 μM in the C. gattii isolate. Thus, we conclude that the laboratory KN99 strain is not intrinsically more sensitive or resistant to HPOs than clinical strains of C. gattii and C. neoformans, even when they are FLC-resistant (Table 1). The same compounds showed a similar trend of inhibition efficacy in C. albicans, with MIC\textsubscript{50} ranging from 1.2 to 50 μM (Table 1). We tested CPO and two effective HPO derivatives in M. furfur and M. pachydermatis and measured MIC\textsubscript{50} of ~25 μM for all three HPOs in both strains (data not shown). Thus, we conclude from these data that the HPOs can show specificity across fungal species.

**HPO SAR against C. neoformans.** Using CPO as a comparator, we explored the R\textsuperscript{4}, R\textsuperscript{5}, and R\textsuperscript{6} positions (marked as 4, 5, and 6 in Figure 1A; Table 1). For CPO, R\textsuperscript{4} = Me, R\textsuperscript{5} = H, and R\textsuperscript{6} = cyclohexyl. Removal of the methyl group from the R\textsuperscript{4} position (9) led to no significant change in the MIC\textsubscript{50} values indicating that R\textsuperscript{4} methyl is nonessential for potency and could be replaced with other moieties that may lead to improved potency in future studies.

Replacement of the R\textsuperscript{6} cyclohexyl ring with a phenyl ring (14a) had a negligible reduction in potency despite a half-log reduction in lipophilicity as indicated by the Clog P value. Extension of the cyclohexyl ring by a methylene (14b) or as a benzyl group (14c) resulted in a minimal reduction in potency. Substitution of the benzyl ring with methoxy groups (16a–c) or 4-chloro (16d) also had a minimal impact on potency as each analog was within two-fold of the potency of CPO. Compounds with a Clog P range of 1.6–2.7 seem to be equally tolerated. These results suggest that the R\textsuperscript{6} position may require a lipophilic group and may tolerate a wide range of substituents, which will be further explored in future studies.

Removal of the R\textsuperscript{6} cyclohexyl ring by moving the lipophilic group as a benzyllic group to the R\textsuperscript{6} position (20) or phenyl to the R\textsuperscript{4} position (25) led to 10- to 30-fold reductions in potency indicating that lipophilicity alone is not a driver of potency and that there is a requirement for a lipophilic group in the R\textsuperscript{6} position.

**HPOs are Fungicides in C. neoformans.** We focused our remaining studies on two of the more effective HPOs SLU-2321 (14b) and SLU-2707 (16d), with SIs of 15 and 18, respectively, and compared them to CPO (Figure 1) in C. neoformans. We assessed their fungicidal properties using a 48 h killing assay. Cells were exposed to CPO, SLU-2321, or SLU-2707 at 0.5×, 1×, 2×, and 4× the MIC for 48 h at 37 °C in a temperature-controlled plate reader with periodic shaking and optical density was measured every hour. The optical density was converted to colony-forming units (CFUs)/mL in Figure 2A–C based on the calibration of the plate reader using hemocytometer cell counts compared to optical density readings. We confirmed the plate reader data by transferring cells to YPD agar plates and incubating at 30 °C for 3 days. Colonies were counted and the number of CFUs was calculated. We observed a log drop in the number of CFUs for cells exposed to 2× the MIC of any of the three compounds (data not shown). This corresponds to the killing of 99.9% of the cells and confirms the fungicidal property of the HPO derivatives, as well as CPO, as had been observed previously in C. neoformans and other fungi.\textsuperscript{5,15,16}

**HPO Derivatives are Additive with AMB and FLC.** We next asked if the HPO inhibition of C. neoformans is altered when combined with the approved antifungal drugs AMB or FLC. This was assessed using a checkerboard assay and the fractional inhibitory concentration index (FICI) with the calculations based on the MIC\textsubscript{50} for the compounds alone and in combination (Table 2).\textsuperscript{23} The combination of CPO or the two derivatives is additive, with an FICI of 0.51. The combinations with AMB were indifferent or additive with FICIs of 0.75 for the combination with CPO and 0.63 for the combination with SLU-2321 or SLU-2707. These data are consistent with a previous study that observed an indifferent or additive effect of the combination of CPO with either AMB or FLC.\textsuperscript{24} Importantly, for future development of HPOs into lead compounds, there was no evidence of antagonism between HPOs and either approved antifungal drug.

**HPOs are Not Antagonistic to Each Other.** Synergy assays can reveal when two compounds inhibit the same enzyme or interfere with each other in some other manner. We compared CPO to SLU-2321 and to SLU-2707 in a checkerboard assay to test for synergy or antagonism between the HPOs. We hypothesize that they will be antagonistic if they are targeting the same enzyme. We observed that CPO showed additive inhibition with SLU-2321 with a FICI = 0.51.

![Figure 1](https://dx.doi.org/10.1021/acsomega.1c00273)
HPO derivatives and, when used in combination, they show additive or synergistic effects. The structure of SLU-2321 is more similar to CPO with a cyclohexylmethylene versus a cyclohexyl at position 5 (Figure 1). In contrast, SLU-2707 has a chlorobenzyl group at the same position so it is plausible that SLU-2707 may target additional or different metalloenzymes than either CPO or SLU-2321. Given that no resistance has been observed for CPO, it is plausible that it targets multiple enzymes, raising the bar to resistance higher than if there is a single enzyme target.

Supplementation with Iron or Copper Can Rescue Inhibition by Some HPOs. We tested whether supplementing the media with iron, copper, or zinc would alter the sensitivity to CPO, SLU-2321, or SLU-2707. We measured the inhibition of cells untreated or treated with CPO (2 μM), SLU-2321 (4 μM), or SLU-2707 (4 μM) in the presence of FeSO₄ from 0.5 to 2 μM. The addition of 0.5 μM iron to the media fully rescued inhibition by any of the three HPOs, strongly suggesting that CPO, SLU-2321, and SLU-2707 act by chelating iron from the active site of enzymes (Figure 3A). Exogenous copper partially rescued inhibition by CPO, but at a higher concentration (4–6 μM) than observed for iron (Figure 3B).

Figure 2. HPOs are fungicidal agents. Cells were treated with increasing doses of (A) CPO, (B) SLU-2321, or (C) SLU-2707 and grown at 37 °C with periodic shaking in a temperature-controlled plate reader. Growth was monitored by optical density measured at 650 nm every hour for 48 h. Error bars represent the standard deviation of the mean of two technical replicates.

Table 2. MICₘₐₜ and FICI for HPOs in Combination with AMB or FLC

| combination     | MICₘₐₜ alone (μM) | MICₘₐₜ combined (μM) | FICI |
|-----------------|------------------|----------------------|------|
|                 | cmpd             | FLC                  | cmpd| FLC | FICI   |
| CPO + FLC       | 3.12             | 3.0                  | 0.05 | 1.5 | 0.51   |
| SLU-2321 + FLC  | 3.12             | 3.0                  | 0.05 | 1.5 | 0.51   |
| SLU-2707 + FLC  | 3.12             | 3.0                  | 0.05 | 1.5 | 0.51   |
| CPO + AMB       | 1.56             | 1.5                  | 0.39 | 0.75| 0.75   |
| SLU-2321 + AMB  | 3.12             | 1.5                  | 0.39 | 0.75| 0.63   |
| SLU-2707 + AMB  | 3.12             | 1.5                  | 0.39 | 0.75| 0.63   |

Figure 3. Metal supplementation can alleviate HPO inhibition in a dose-dependent manner. Cells treated with 2 μM CPO, 4 μM SLU-2321, or 4 μM SLU-2707 were supplemented with no or increasing concentrations of (A) FeSO₄ from 0.13 to 1 μM, (B) CuSO₄ from 2 to 10 μM, or (C) ZnSO₄ from 2 to 10 μM. Growth was measured after 48 h at 35 °C and normalized to the growth of vehicle-treated control cells. Error bars represent the standard deviation of the mean of three technical replicates. This graph is representative of two more experiments conducted on different days.
Table 3. MIC$_{a0}$ and FICI for HPOs in Combination with Metal Chelators

| combination                  | alone (µM) | MIC$_{a0}$ | combined (µM) | FICI |
|------------------------------|------------|------------|---------------|------|
| CPO + EDDHA                  | 1.56       | 0.39       | 0.78          | 0.31 |
| SLU-2321 + EDDHA             | 3.12       | 0.39       | 0.78          | 0.19 |
| SLU-2707 + EDDHA             | 3.12       | 0.19       | 0.78          | 0.12 |
| CPO + DFP                    | 1.56       | 0.05       | 50            | 0.28 |
| SLU-2321 + DFP               | 1.56       | 0.05       | 50            | 0.28 |
| SLU-2707 + DFP               | 3.12       | 0.05       | 50            | 0.26 |
| CPO + DFO                    | 1.56       | 0.39       | 3.12          | 0.26 |
| SLU-2321 + DFO               | 3.12       | 0.19       | 3.12          | 0.07 |
| SLU-2707 + DFO               | 3.12       | 0.39       | 3.12          | 0.14 |
| CPO + neocuproine            | 1.56       | 0.05       | 0.125         | 0.53 |
| SLU-2321 + neocuproine       | 1.56       | 0.05       | 0.125         | 0.53 |
| SLU-2707 + neocuproine       | 3.12       | 0.05       | 0.125         | 0.53 |
| CPO + BCS                    | 1.56       | 1.56       | >200          | 2    |
| SLU-2321 + BCS               | 3.12       | 3.12       | >200          | 2    |
| SLU-2707 + BCS               | 3.12       | 3.12       | >200          | 2    |

*EDDHA is a selective iron chelator. *DFP is a selective iron chelator. *DFO is a selective iron chelator. *Neocuproine is a selective copper(i) chelator. *BCS is a selective copper chelator.

3B). However, copper only partially rescued inhibition by SLU-2321 at 10 µM and did not rescue inhibition by SLU-2707 at any of the tested concentrations (Figure 3B). The addition of zinc up to 10 µM did not rescue the inhibition by any of the three HPOs tested (Figure 3C). This suggests that CPO may target both Fe- and Cu-dependent enzymes whereas SLU-2321 and SLU-2707 primarily target Fe-dependent enzymes. It is possible that the presence of copper also induces wide-spread transcriptional changes that alter the metabolism and alter the expression of iron-related enzymes.25

We used BD Difco YNB without amino acids as our base media and it is reported to contain copper sulfate (40 µg/L or 250 nM), ferric chloride (200 µg/L or 1.2 µM), and zinc sulfate (400 µg/L or 2.5 µM). C. neoformans has multiple iron transporters and it is unknown how much of the metal ions are transported into the cells after 48 h of growth under these conditions.26,27 For iron, we added up to 2 µM exogenous iron, which is likely similar to the concentration in the media at the start of the assay. The exogenous levels of copper and zinc are significantly higher than the reported concentration of either metal ion in the media. It is unlikely that the water contributes significant levels of metal ions as the media is mixed in water from a Milli-Q IQ system with both reverse osmosis and ion exchange membranes that remove all ions down to trace levels according to the system documentation.

**Addition of Exogenous General Metal Chelators Increases Sensitivity to HPOs.** We examined the effect of different metal chelators on the inhibition of cells by CPO, SLU-2321, and SLU-2707. We measured the MIC$_{a0}$ values for two different copper chelators, bathocuproinedisulfonic acid (BCS) and neocuproine and three iron chelators, ethylenediamine-N,N’-bis(2-hydroxyphenylacetic acid) (EDDHA), deferoxamine mesylate (DFO), and deferiprone (DFP) to test for their inhibition of C. neoformans alone. At BCS concentrations up to 200 µM, we saw no inhibition, whereas the neocuproine was able to significantly inhibit cell growth with an MIC$_{a0}$ of 0.25 µM (Table 3). Of the three iron chelators tested, only EDDHA showed any inhibition, with an MIC$_{a0}$ of 12.5 µM, whereas DFO and DFP did not inhibit cell growth at concentrations up to 200 µM (Table 3). We then tested the chelators for potential synergistic activity with the HPOs using a checkerboard assay. Neocuproine showed an additive effect with all three HPOs, with an FICI of 0.53, whereas BCS showed no interaction with the HPOs (Table 3). We observed strongly synergistic effects with all three iron chelators in combination with CPO, SLU-2321, or SLU-2707 with FICIs between 0.07 and 0.31 for the combinations (Table 3). These data indicate that all three HPOs primarily inhibit by chelation of iron. The much higher MIC$_{a0}$ for the iron chelators in the absence of HPOs suggest that general iron chelation from the media does not explain the strong inhibition of cryptococcal growth by HPOs. These data, together with the SAR data demonstrating that not all HPO are inhibitory, strongly suggest that effective inhibitors bind to one or more metalloenzymes and chelate the Fe atoms within the active site, similar to the mechanism of drugs that target HIV integrase or HBV RNaseH.

**Mitochondrial Inhibitors Increase Sensitivity to HPOs.** There are more than 100 proteins in the Cryptococcus genome that are either iron-dependent or involved in regulation and metabolism of iron. This estimate is based on the hypothesis that 1–2% of eukaryotic genomes encode Fe-dependent proteins,30,31 a recent analysis of iron metabolism in Saccharomyces cerevisiae that identified 139 Fe-containing proteins,32 and unpublished bioinformatic analyses of the C. neoformans proteins (MJD). Iron-dependent enzymes play key roles in most metabolic processes and are significantly enriched in mitochondria processes, particularly in enzymes that form the electron transport chain (ETC). Therefore, we examined the impact of the HPOs on mitochondrial function using synergy assays with six different inhibitors of the ETC including: rotenone, which targets complex I; malonate,
which inhibits complex II; salicylhydroxamic acid or SHAM, which inhibits the alternative oxidase; antimycin-A and myxothiazol, both of which inhibit complex III; and potassium cyanide, which inhibits complex IV. The ETC inhibitors were used in checkerboard assays with the three HPOs to determine if they alter the inhibition of C. neoformans cells. We observed a strongly synergistic interaction between the HPOs and rotenone, SHAM and KCN, whereas the interaction with malonate, antimycin-A, or myxothiazol showed an additive or indifferent response (Table 4). Because the HPOs are synergistic with more than one inhibitor of the ETC, it suggests inhibition of multiple Fe-dependent enzymes that are either directly involved in ETC or are important for production of Fe-dependent enzymes that form the ETC.

HPOs Do Not Alter Respiration in Human Liver-Derived Cells. We measured the mitochondrial respiration of human hepatoma cells using a Seahorse assay after a one-hour preincubation with CPO, SLU-2321, SLU-2707, and SLU-2239. SLU-2239 was included as a control as it did not inhibit fungal growth and had the highest CC₅₀ value of the four compounds (Table 1). Of the four HPOs, only SLU-2707 had a measurable impact on human mitochondrial respiration (Figure 4). This suggests that the toxicity to mammalian cells is unlikely to be a function of mitochondrial toxicity. However, it is possible that the HPOs could alter mitochondrial respiration in fungi, whose ETC has an alternative oxidase, encoded by AOX1, which acts as an additional electron-receptor. We measured the MIC₅₀ for CPO and the two HPO derivatives in an aox1Δ strain but observed no difference in sensitivity between the deletion strain and wild type (data not shown). Thus, it is unlikely HPOs act directly on the Aox1 protein but does not rule out inhibition of other fungal mitochondrial proteins or processes.

## DISCUSSION

This study was designed to identify the basic SAR relationships relative to efficacy of CPO derivatives against C. neoformans and to identify tractable chemical avenues for chemical
optimization. We screened 10 novel HPO derivatives of the approved topical antifungal drug, ciclopirox (CPO), for inhibition of *C. neoformans*, *C. gattii*, and *C. albicans*. Eight of the 10 had MIC<sub>90</sub> below 2 μM in *C. neoformans*, comparable to CPO in our assays. In *C. neoformans*, the HPOs are fungicides and showed an additive interaction with the current antifungals, AMB and FLC. They are synergistic with multiple mitochondrial ETC inhibitors and most likely act by chelating iron in the active site of iron-dependent enzymes. By providing this preliminary SAR, this study provides a starting point for a medicinal chemistry campaign to develop the HPOs into lead candidates for treatment of cryptococcal and other systemic fungal infections.

### Possible Mechanisms of Action of HPOs

CPO was approved as a topical antifungal therapeutic agent more than 30 years ago, but the mechanism of action is still not well understood in fungi. We chose two derivatives and CPO to further explore the biology of the HPOs in *C. neoformans* (Figure 1). We examined the impact of divalent metal chelators on the sensitivity of *C. neoformans* to HPO inhibition and saw a synergistic interaction with three different iron chelators, EDDHA, DFO, and DFP, but not with two copper specific chelators (Figure 3). Adding exogenous iron to the media abrogated inhibition by all three HPOs, whereas adding exogenous copper abrogated inhibition by CPO but did not alter the inhibition by the other two HPOs. Exogenous zinc had no effect on inhibition. Thus, we conclude that all three of these HPOs are targeting one or more iron dependent enzymes. It is possible that the excess iron or copper competes with the active site iron for binding to the CPO analog, thereby reducing the concentration of free CPO-analog to bind and inhibit the Fe-dependent enzyme. This may also explain why copper can partially rescue the effect of CPO, but at a much higher concentration. CPO and its analogs likely target more than one enzyme because CPO in combination with either SLU-2321 or SLU-2707 showed an additive or synergistic interaction, rather than an antagonistic interaction that would be expected if all three compounds targeted the same enzyme.

### Interaction of HPOs with Mitochondrial ETC Inhibitors

Rotenone targets complex I of the ETC, which is comprised of 12–14 core subunits and up to 30 accessory proteins, whose number varies across species. Rotenone binds to the ubiquinone binding pocket of the 49 kDa subunit of *C. neoformans* (Figure 1). In *Yarrowia lipolytica*, Synergy with this inhibitor suggests that the HPOs most likely do not directly target the same binding pocket of that subunit but may bind allosterically to the 49 kDa subunit or inhibit other proteins within complex I. SHAM inhibits the alternative oxidase, which can accept electrons directly from the quinone pool and bypass complex III. However, we found no difference in MIC<sub>90</sub> for HPOs in an *aox1Δ* strain deletion strain versus wild-type, so the Aox1p is unlikely to be a direct target of the HPOs. It is possible that either direct inhibition of one or more Fe-S cluster proteins associated with complex I or disruption of Fe-dependent enzymes by HPOs may affect the biogenesis of these proteins and disrupt the ETC chain at multiple points. This would likely cause an increase in reactive oxygen species, which may explain the synergy with SHAM inhibition of Aox1p, which has a role in protection against oxidative stress. The synergy with KCN may be explained if the HPOs are disrupting one or more proteins involved in steps leading to complex IV, rendering the cell more vulnerable to further disruption of the ETC. The reduced synergy of HPOs with complex II and complex III inhibitors may reflect the alternate pathway for electron transport that can bypass complex II or, with a functional alternative oxidase, a second terminal electron receptor can function in the absence of complex III.

### CONCLUSIONS

The rate of development of new antifungal therapies is not meeting the needs of coping with an increasing number of invasive fungal infections. The HPOs are a promising scaffold for development into novel antifungal agents. Here, we present an initial SAR that defines a set of structural requirements for potency of these compounds and provides guidance for future optimization through a medicinal chemistry campaign. We have shown their efficacy in three fungal species and established that inhibition of iron-dependent enzymes is the most likely mechanism of action. These data establish a starting point to develop novel compounds that are more potent and selective, identify the enzymatic target(s) of this scaffold, and develop CPO derivatives suitable for systemic administration. Future studies are needed to address the bioavailability of HPOs, their ability to cross the blood–brain barrier, and their efficacy in animal models of infection.

### EXPERIMENTAL SECTION

See Supporting Information for the synthesis and characterization of compounds used in this study.

#### Strains, Media, and Chemicals

*KN99α*, a strain of *C. neoformans* serotype a, was used as the wild-type strain and was obtained from Dr. Jennifer Lodge, Washington University. The *C. neoformans*, DUMC-158.03, and *C. gattii*, RSA-3615, clinical strains were obtained from Dr. John Perfect, Duke University. The deletion strain for the *AOX1* gene is from the Madhani 2015 gene deletion library and was obtained from the Fungal Genetics Stock Center. *C. albicans* (Robin) Berkhoustrain (ATCC #90028), *M. furfur* (Robin) Baillon (ATCC #14521), and *M. pachydermatis* (Wiedman) Dodge, anamorph (ATCC #14522) were purchased from the ATCC. *C. neoformans*, *C. gattii*, and *C. albicans* strains were grown on YPD (1% yeast extract, 2% bacto-peptone, and 2% dextrose). Solid media contained 2% bacto-agar. Selective YPD medium contained 100 μg/mL nourseothricin (GoldBio, USA) and/or 200 μg/mL G418 (Genetec; Gibco Life Technologies, USA). YNB-02 (0.67% yeast nitrogen base, 0.2% dextrose, pH 7.0 with 50 mM MOPS) was used for all limiting dilution inhibition assays unless otherwise noted. The media prepared in deionized water passed through an ion exchange Milli-Q filter system (Sigma-Millipore, USA). *M. furfur* and *M. pachydermatis* were grown on mDixon [3.5% malt extract (Oxoid L39), 0.6% bacto-tryptone, 2% ox-bile (Oxoid L50), 1% tween-40, 0.2% oleic acid, and 0.2% glycerol].

DME is Gibco Dulbecco’s modified Eagle medium (Thermo Fisher Scientific, Waltham MA, USA). DME:F-12 is Dulbecco’s modified Eagle medium/nutrient mixture F-12 1:1 (Cytiva, Washington, D.C., USA) with 10% FBS (fetal bovine serum) and 1% P/S (penicillin/streptomycin) and was used for the metabolic flux assays.

Stocks of AMB, FLC, and CPO (Selleck Chemicals, Houston, TX, USA) were prepared at 10 mM in dimethyl sulfoxide (DMSO) and stored at −20 °C. Stocks of zinc sulfate, cupric sulfate, iron chloride, DFO, DFP, BCS, malonic acid, and potassium cyanide (Millipore-Sigma, St. Louis, MO) were prepared in Milli-Q water and filter sterilized before
addition to media. EDDHA (Thermo Fisher Scientific, Waltham MA, USA) was prepared in Milli-Q water and filter-sterilized before addition to the media. Neocuproine, myxothiazol, and rotenone (Millipore-Sigma, St. Louis, MO) were prepared in DMSO and filter sterilized before addition to the media.

**Determination of MIC.** The Cryptococcus and C. albicans cells were grown overnight in 4 mL cultures of YPD at 30 °C with shaking and then diluted to with an optical density (650 nm) of 0.001 in YNB-02 + 1% DMSO for the limiting dilution assay, which has been identified as an appropriate substitute for C. neoformans susceptibility testing. The compound dilution series and cells were incubated in round-bottom clear 96-well plates without shaking for 48 h at 35 °C and the optical density measured. Malassezia cells were grown overnight in 7 mL cultures of mDixon on 30 °C with shaking and diluted in the same media to OD_{650} = 0.05 for the assay. The compound dilution series and cells were incubated in a final volume of 300 μL in a 96-well deep well plates for 48 h with shaking at 30 °C. Resazurin was added to each well at a final concentration of 0.01% resazurin and incubated with the cells for 2 to 3 h at 37 °C. The cells were centrifuged, and the supernatant was transferred to a 96-well clear bottom plate. The fluorescence level was read at an excitation of 560 nm and emission of 590 nm.

The MIC was determined using compound concentrations from 0.19 to 50 mM, unless otherwise noted, in YNB-02 + 1% DMSO. Each assay was done in triplicate and all values are the average of two or more independent assays. The data are presented as the average cell density as a percentage of DMSO-only treated cells. MICs are reported as the minimal concentration needed to inhibit 80% of fungal cell growth relative to vehicle-treated controls.

**Determination of Drug–Drug or Drug–Chelator Interactions.** Drug–drug interactions were assessed using the checkerboard assay. The MICₘₐₓ of compounds CPO, SLU-2321, and SLU 2707 were measured using compound concentrations from 0.19 to 12.5 μM and MICs for FLC and AMB were measured using concentrations from 0.08 to 12.5 μM. Each assay was performed in triplicate and all values are the average of two or more independent assays. The data are presented as the average cell density as a percentage of DMSO-only treated cells. MICs are reported as the minimal concentration needed to inhibit 80% of fungal cell growth relative to vehicle-treated controls.

**Cytotoxicity in Human Hepatoma Cells.** Huh7 cells were seeded in 96-well plates at 1.0 × 10⁴ cells per well and incubated for 48 h in Dulbecco’s modified Eagle’s medium (DMEM):F-12 2.5 mM L-glutamine, 15 mM HEPES supplemented with 10% FBS and 1% P/S. Test compounds were applied to the cells at concentrations ranging from 0.19 to 50 μM in the presence of 1% DMSO for 72 h. Cytotoxicity was measured using the CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay (Promega, USA). Briefly, a 2 mg/mL [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt] and 0.043 mg/mL phenazine methosulphate solution was added to the medium and incubated at 37 °C for 2 hours then the absorbance was measured at 490 nm. The data were calculated using the four-parameter variable slope log(inhibitor)-versus-response algorithm with the bottom set to zero with GraphPad Prism (v8, www.graphpad.com). The concentration at which 50% of cells were inhibited relative to vehicle-treated control is reported as the CC₅₀ value.

**Killing Assays.** We conducted a time-course of exposure of wild-type KN99 cells at a starting optical density of 0.001 (~1 × 10⁴ cells/mL). Replicate wells were incubated at 37 °C for 48 h in a shaking plate reader (BioTek Synergy HTX, BioTek Instruments, USA) with CPO, SLU-2321, and SLU 2707 at 0.5, 1, 2, and 4 μM. The optical density at 650 nm was measured every hour. In a second assay, wild-type KN99 cells were used in a limiting dilution assay with CPO, SLU-2321, and SLU 2707 using a concentration range from 0.19 to 50 μM and incubated at 37 °C for 48 h. The conversion of optical density readings to CFU was determined using hemocytometer cell counts at different OD₆₅₀ readings.

**Media Supplementation Assays.** Three replicate cultures of KN99 cells were incubated with 2 μM CPO, 4 μM SLU-2321, or 4 μM SLU 2707 in the presence of increasing concentrations of metal ions for 48 h at 35 °C in YNB-02 + 1% DMSO. The percent inhibition was measured relative to the DMSO only control. For iron, iron sulfate (II) was added at 0.5, 1, and 2 μM to the cells. For copper supplementation, copper (II) sulfate was added at 1, 2, and 4 μM. For zinc supplementation, cells were incubated with 2, 4, 6, 8, and 10 μM zinc sulfate. Each experiment was carried out in triplicate and the values represent the average of two or more independent replicates.

**Mitochondrial Stress Test Using Agilent Seahorse XFP Flux Analyzer.** Human hepatoma Huh7 cells were cultured in DMEM:F-12 with 10% FBS, 1% P/S, and plated in an XFP Cell Cartridge plate (Agilent, Santa Clara, CA) at 16,000 cells (80 μL) per well and were incubated overnight at 37 °C in 5% CO₂ incubator. Huh7 cells were washed once with XF assay media (Agilent) supplemented with glucose (16.9 mM), i-glutamine (2.5 mM), and sodium pyruvate (0.5 mM) at pH 7.4, which was prewarmed to 37 °C and incubated at 37 °C in a non-CO₂ incubator with HPO compounds (5 μM) or DMSO for one hour. The oxygen consumption rate (OCR) was measured using the Agilent Seahorse XFP Analyzer and mitochondrial reparation was assessed by sequential addition of 1.5 μM oligomycin (Agilent); 0.5 μM fluoro-carbonyl cyanide phenylhydrazone (Agilent); and 0.5 μM rotenone and antimycin A (Agilent). The data were collected at the indicated times and processed using Prism (GraphPad, La Jolla, CA).
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Notes

The authors declare no competing financial interest.

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