1 **Occidiofungin, an actin binding antifungal with *in vivo* efficacy in a vulvovaginal**
2 **candidiasis infection**

3 **Short Title: Occidiofungin, an actin binding antifungal**

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Abstract

Current antifungal treatment options are plagued with rapidly increasing occurrence of resistance, high degree of toxicity and a limited spectrum of activity. The need to develop a novel antifungal with a unique target, wider spectrum of activity, and reduced toxicity to the host, is urgent. We have identified and characterized one such compound named occidiofungin that is produced by the soil bacterium *Burkholderia contaminans* MS14. This study identifies the primary cellular target of the antifungal, which was determined to be actin. Actin binding metabolites are generally characterized by their ability to inhibit polymerization or depolymerization of actin filaments, which presumably accounts for their severe toxicity. Occidiofungin, instead, has a subtler effect on actin dynamics that triggers apoptotic cell death. We were able to demonstrate the effectiveness of the antifungal in treating a vulvovaginal yeast infection in a murine model. This discovery puts occidiofungin in a unique class of actin-binding antifungal compounds with minimal reported toxicity to the host. The results of this study are important for the development of a novel class of antifungals that could fill the existing gap in treatment options for fungal infections.

Author summary

Widespread resistance to antifungal compounds currently in use has been alarming. Identification and development of a new class of antifungals with a novel cellular target is desperately needed. This study describes the assays carried out to determine the molecular target and evaluate efficacy of one such novel antifungal compound called occidiofungin. Occidiofungin modified with a functional alkyne group enabled affinity purification assays and localization

43   studies in yeast. These studies led to the identification of the actin binding property of

44   occidiofungin. Actin-binding by secondary metabolites often exhibit severe host toxicity, but this

45   does not appear to be the case for occidiofungin. We have previously been able to administer

46   occidiofungin to mice at concentrations in the range of 5 mg/kg without any serious complications.

47   We were able to demonstrate the effectiveness of the antifungal in treating a vaginal fungal

48   infection in a murine model. The results outlined in this manuscript establish that occidiofungin is

49   an efficacious compound with a novel molecular target, putting it in a completely new class of

50   antifungals.

51

Introduction

53   Fungal infections caused by pathogens that are resistant to commonly used classes of

54   antifungals are becoming increasingly prevalent. Recently, a CDC report described the spread of

55   multi-drug resistant *Candida auris* causing systemic infections in hospitalized patients [1].

56   Furthermore, other species such as *Candida glabrata* and *Candida parapsilosis* have been reported

57   to have gained resistance to routinely used azoles and echinocandins [2-4]. An example of a fungal

58   infection that is rapidly developing resistance to currently available forms of treatment is

59   vulvovaginal candidiasis (VVC). VVC will affect approximately 75% of all women and 5-10%

60   of all women will develop recurrent VVC (RVVC) [5-7]. Approximately 90% of VVC is caused

61   by *C. albicans*, while the remaining 10% is caused by *C. glabrata*, *Candida tropicalis*, *Candida*

62   *parapsilosis,* and *Candida krusei*. These non-albicans VVC causing species are generally resistant

63   to azole treatments, which are the most common treatment option for VVC [3, 8]. There have been

64   no new therapeutic developments in decades for recurrent VVC. In the absence of well-established

65   medical treatment standards, several ineffective methods for treating VVC have been prescribed,

66   which generally include the use of a rigorous dosing regimen of antifungals followed by a long

67   period of prophylactic dosing [5, 9, 10].

68   *Candida* species are an important cause of infection and mortality in all hospitalized

69   patients[11]. Candidemia has a mortality rate of 30%–50% in cancer patients and is a major

70   complicating factor for the successful treatment of cancer. Despite current antifungal drugs,

71   invasive fungal infections are still a major cause of morbidity and mortality in transplant patient

72   population[12, 13]. Reports suggest that candidal infection is the first and second most common

73   infection in lung and heart transplant recipients, respectively[14-17]. In heart transplant recipients,

74   candidal disease has been attributed to a mortality rate of 28%[18]. A rise in candidemia caused

75   by non-albicans *Candida* spp. and an increase in azole resistance[19-23] is alarming; and supports

76   the need for new antifungals. This problem is expected to be exacerbated by the presence of the

77   multidrug resistant fungi *Candida auris* in hospitals. The increase in *C. auris* infections is expected

78   to further reduce the positive therapeutic outcomes associated with currently approved antifungals

79   [21]. Additionally, more appropriate antifungal treatment options may reduce the cost of treatment

80   and mortality of patients.

81   Clinically approved antifungals primarily comprise members of the polyene, echinocandin,

82   and azole family of compounds. The polyene antifungal amphotericin B was introduced in the

83   1950s and was the only antifungal available until the introduction of the azole class of antifungals

84   in the 1980s. These two groups primarily target ergosterol production or bind to ergosterol,

85   disrupting the fungal membrane. The echinocandins, the third group, are synthetically modified

86   lipopeptides that originate from a natural cyclic peptide compound produced by fungi. This group

87   selectively inhibits 1,3-β-glucan synthesis by functioning as a non-competitive inhibitor of 1,3-β-

88   glucan synthase [24-27]. Widespread resistance and the ineffective spectrum of activity of this

4

89 class of antifungals have been reported [28-32]. The prevalence of echinocandin- and azole-

90 resistant fungal pathogens and the limited spectrum of activity of those compounds is one major

91 issue contributing to the need for a new class of antifungals. Additionally, current antifungal

92 treatments lead to abnormal liver and kidney function tests and have limitations with respect to

93 their spectrum of activity and toxicities [33, 34]. Presumably, the identification of a novel class of

94 antifungals with a broad spectrum of activity and a unique mechanism of action would mitigate

95 the loss of life associated with the use of the current classes of antifungals. These limitations and

96 toxicity problems have created an urgent need to identify antifungal compounds that have a novel

97 mechanism of action [35].

98   Occidiofungin is a non-ribosomally synthesized glycolipopeptide produced by the soil

99 bacterium *Burkholderia contaminans* MS14 [36]. It is a cyclic peptide with a base mass of 1200

100 Da (Fig 1). The bacterium produces a mixture of structural analogs of the base compound

101 (occidiofungins A-D), however all analogs are composed of eight amino acids and a novel C18

102 fatty amino acid (NAA) containing a xylose sugar, and a 2,4- diaminobutyric acid (DABA). The

103 structural analogs differ by an addition of oxygen to asparagine 1 (Asn1) forming a β-hydroxy

104 asparagine 1 (BHN1) and by the addition of chlorine to β-hydroxy tyrosine 4 (BHY) forming 3-

105 chloro β-hydroxy tyrosine 4 (chloro-BHY) [36]. Occidiofungin has a wide spectrum of activity

106 against filamentous and non-filamentous fungi and minimal toxicity in an animal system [36, 37].

107 We have previously demonstrated that the mechanism of action of occidiofungin differs from the

108 primary mode of action of the three common classes of antifungals [38, 39]. Briefly, there was no

109 decrease in the activity of occidiofungin against *C. albicans* in the presence of 0.8 M sorbitol, an

110 osmotic stabilizer, indicating that occidiofungin was not causing osmotic stress by cell wall or

111 membrane disruption (the primary mechanism of action of azoles). Further, upregulation of

112   Hog1p, which is an osmotic disruption indicator, was significantly lower than that seen for

113   conditions known to induce the osmotic stress response pathway (e.g. 1 M NaCl). Mutants lacking

114   Fks1p, an enzyme in the cell wall biosynthesis pathway, did not demonstrate increased resistance

115   to occidiofungin. Disruption of the Fks1/Fks2 complex is the primary mechanism of action of

116   echinocandins. Additionally, the introduction of vesicles containing ergosterol, the target of

117   amphotericin, did not reduce the activity of occidiofungin unlike the case with amphotericin B.

118   When observed under a microscope, occidiofungin-treated cells did not undergo lysis but appeared

119   shrunken in size. Additional assays indicated that occidiofungin rapidly induces apoptosis in yeast

120   cells at the minimum inhibitory concentration [39]. Interestingly, a critical threshold concentration

121   of occidiofungin is required for its observed fungicidal activity. Occidiofungin has little impact on

122   the growth rate of yeast at sub-inhibitory concentrations [38]. In addition, occidiofungin was seen

123   to have potent inhibitory activity against *Pythium* species which lacks ergosterol in the membrane

124   and against *Cryptococcus neoformans* which is resistant to echinocandins [36]. Preliminary

125   toxicological analyses of occidiofungin using a murine model indicated that it was well tolerated

126   at concentrations of 10 to 20 mg/kg [37]. Intravenous administration of occidiofungin to mice at a

127   dose of 5 mg/kg was carried out with minimal induced toxicity. Blood chemistry analyses and

128   histopathology performed on multiple organs showed a transient non-specific stress response with

129   no damage to organ tissues [40]. Taken together, the data suggest that occidiofungin is a promising

130   candidate for development as a clinically useful antifungal agent. This report describes studies to

131   identify the molecular target of occidiofungin and determine its efficacy in a murine model of

132   vulvovaginal candidiasis.

133   Fig 1. Covalent structure of occidiofungin A-D and alkyne-OF.

134   **Results**

6

Spectrum of activity of occidiofungin against clinically relevant fungi.

135

136 Occidiofungin causes cell death in fungi through a mechanism of action that is distinct

137 from the clinically used classes of antifungals [39]. Due to its unique mechanism of action,

138 occidiofungin has sub-micromolar activity against azole and echinocandin resistant strains of

139 fungi. Strains of *Candida albicans, Candida glabrata, and Candida parapsilosis* that were

140 resistant to fluconazole and caspofungin were sensitive to occidiofungin (S1 Table). Furthermore,

141 strains of *C. auris* were sensitive to occidiofungin at sub-micromolar concentrations. Non-albicans

142 strains are believed to be the primary cause of recurrent vulvovaginal candidiasis. Strains of

143 *Candida parapsilosis* and *C. neoformans* that were resistant to treatment with caspofungin were

144 found to be susceptible to treatment with occidiofungin. Occidiofungin was also found to be active

145 against *Aspergillus, Mucor, Fusarium,* and *Rhizopus* species. Several strains of the dermatophyte

146 *Trichophyton* were also found to be susceptible to occidiofungin treatment, including azole and

147 terbinafine resistant strains. A summary of the results, as reported in S1 Table, indicate that

148 occidiofungin has activity against filamentous and non-filamentous fungi at sub-micromolar

149 concentrations and has a broader spectrum of activity compared to other clinically available

150 antifungals. Furthermore, sensitivity of fungal strains resistant to azoles and echinocandin class of

151 antifungals support the notion that occidiofungin is functioning via a novel mechanism of action.

Perturbation of actin-based functions following occidiofungin exposure.

152

153 As a dimorphic fungus, *C. albicans* can grow as yeast or hyphae and the ability to switch

154 between these forms is linked to the pathogenicity of the organism [41]. As most studies on

155 occidiofungin efficacy have been carried out on *C. albicans* in their yeast form, the impact of the

156 antifungal on morphological switching was tested. Incubation of *C. albicans* with sub-inhibitory

157 concentration of occidiofungin was shown to block hyphae formation in cells that were induced to

158   undergo morphological switching (Fig 2). Morphogenesis of *C. albicans* from yeast to filamentous

159   forms has been shown to involve actin dynamics as treatment with cytochalasin A, latrunculin A,

160   or the elimination of myosin I function prevent hyphae formation [42, 43]. In *C. albicans*,

161   maintenance of the actin scaffold is also necessary for endocytosis, DNA segregation, and cell

162   division [44, 45]. To determine whether occidiofungin impacts other cellular activities linked to

163   actin dynamics, the effect of occidiofungin on endocytosis in fission yeast was evaluated by

164   staining cells with FM-464 (Fig 3). Cells exposed to 0.5X MIC and 1X MIC demonstrated a

165   concentration dependent reduction in stained endocytic vesicles. Actin has also been linked to the

166   proper positioning of the mitotic spindle during cell division, and mutants that lack actin cables

167   have been shown to accumulate multinucleated cells [46, 47]. Within thirty minutes of exposure,

168   both *S. cerevisiae* and *C. albicans* cultures treated with a sub-inhibitory concentration of

169   occidiofungin were found to accumulate a low percentage of binucleated cells indicative of a

170   disruption or a delay in nuclear transit through the mother-daughter neck (S2 Table). To further

171   characterize the role of actin in cellular response to occidiofungin, we analyzed haploid *S.*

172   *cerevisiae* mutants deleted for genes linked to actin polymerization and depolymerization. Of the

173   eighteen strains tested, only the Δ*tpm1* mutant showed altered sensitivity to occidiofungin, with

174   the deletion mutant exhibiting a four-fold resistance to occidiofungin [S3 Table]. The observed

175   increase in resistance to occidiofungin in the absence of the *tpm1* gene, which codes for the major

176   isoform of tropomyosin, may be due to the mutant's increased tolerance of cellular stressors

177   (unpublished data) or a decrease in cellular growth rate [48]. A decrease in cellular growth has

178   previously been linked to occidiofungin resistance [49]. The lack of an observed effect on

179   occidiofungin activity with the vast majority of the major actin associated proteins suggests that

180   they are not directly involved in the observed inhibitory activity of occidiofungin.

181 Fig 2. *Candida albicans* morphology under hyphae inducing conditions. (A) The resulting
182 morphology was scored as either 'yeast' or 'filamentous' at two hours and the resulting percent
183 given. The data is presented as the average with the standard deviation for over 200 cells from
184 each treatment condition (n=3). (B) The resulting cell morphology was scored as either 'yeast' or
185 'filamentous' after 0, 1, 2, 4, and 6 hours at 37°C. The data is presented as the average with the
186 standard deviation for over 200 cells from each treatment condition (n=3). DMSO treated samples
187 are represented by triangles; Occidiofungin treated samples represented by circles.

188 Fig 3. Effect of the native occidiofungin on endocytosis in fission yeast. DIC (top row) and
189 fluorescence (bottom row) images of cells stained using FM-464 following treatment with sample
190 blank (left column), 0.5X MIC of occidiofungin (middle column), and 1X MIC occidiofungin (last
191 column). FM-464 dye uptake by endocytosis decreases in cells exposed to occidiofungin a dose
192 dependent fashion.

193 **Alkyne derivatization of occidiofungin.**

194 In order to localize occidiofungin in yeast and to identify its cellular binding partners,

195 methods to fluorescently label or add a functional purification tag to occidiofungin were needed.

196 To this end, occidiofungin was chemically modified with a terminal alkyne through acylation of

197 the free amino group of the diaminobutyric acid residue at position 5 (S1 Figure) for subsequent

198 click chemistry (Sharpless-Hüisgen cycloaddition). Structural analysis of the derivatized product

199 (S2 Figure and S3 Figure) revealed that occidiofungin (OF-B) and burkholdine (Bk-1215)[50],

200 isolated by Schmidt, are likely identical products. The modified occidiofungin, alkyne-OF, had an

201 eight-fold reduction in activity with the minimum inhibitory concentration of 1 and 0.5 µg/mL

202 against *Saccharomyces cerevisiae* BY4741 and *Schizosaccharomyces pombe* 972 h-, respectively

203 (S4 Table). To determine whether alkyne-OF still had the same apoptosis inducing bioactivity as

204 the native occidiofungin, *S. cerevisiae* was treated with alkyne-OF and apoptotic assays such as

205 TUNEL, reactive oxygen species (ROS) detection, and phosphatidylserine externalization assays

206 were performed. Double stranded DNA breaks, the generation of ROS, and the externalization of

207 phosphatidylserine were observed in the alkyne-OF treated cells, supporting the same mechanism

208   of action (S4 Figure A-C). Although this alkyne modification moderately reduced the inhibitory

209   activity of the compound, the functionalized derivative has the same apoptotic bioactivity and was

210   therefore used to identify the fungal target.

211

212   **Identification of occidiofungin interacting proteins.**

213   Alkyne-OF was used in a pull-down assay to identify intracellular proteins that directly or

214   indirectly interact with occidiofungin (Fig 4A). In brief, alkyne-OF was incubated with *S.*

215   *cerevisiae* cells and the resulting cell lysates subsequently reacted with biotin-azide with

216   occidiofungin-interacting proteins captured by passage over streptavidin beads. In the SDS PAGE

217   gel, samples that included alkyne-OF had a more pronounced Coomassie blue stained band of

218   captured proteins compared to control samples (Fig 4A). These bands were removed from the SDS

219   PAGE gel for subsequent LC-MS/MS analysis. A silver stained gel containing samples

220   electrophoresed to completion is provided to further show that the alkyne-OF was more efficient

221   at capturing proteins in the pull-down assay. Data from multiple analyses using *S. pombe* 972h-

222   and *S. cerevisiae* BY4741 were pooled. The resulting list of proteins obtained following LC-

223   MS/MS analysis of excised bands was distilled as follows. Proteins that were observed in the two

224   control samples, DMSO treated and native occidiofungin treated, were removed from

225   consideration resulting in proteins that were exclusively found in the test sample captured with the

226   alkyne-OF variant (S5 Table). The culled protein list was grouped based on gene ontology

227   including cellular localization and/or molecular function. The resulting distribution is presented in

228   Fig 4B. This analysis revealed that the majority of the proteins pulled down by alkyne-OF were

229   actin or actin associated proteins (e.g. Pil1 and Cap1). In addition to actin-related proteins, proteins

230   involved in vesicle transport and mannosylation were found associated with alkyne-OF. The

231 remaining proteins were ribosomal and mitochondrial related proteins. The data indicates that

232 occidiofungin plays a role in binding to actin since a majority of the proteins either directly

233 influence actin dynamics (e.g. Arp2/3), are in close proximity to actin patches within the cell, or

234 utilize actin filaments for their activity (e.g. Myo1).

235 Fig 4. Determination of *in vivo* interaction of occidiofungin. A) Representative samples obtained
236 following affinity purification of whole cell extracts run on 12% SDS PAGE gels and stained with
237 Coomassie blue (top) and silver staining (bottom). The Coomassie stained gel was run only until
238 the proteins entered the separating gel whereas the silver stained gel was allowed to run
239 completely. The bands in the Coomassie stained gel (demarcated by the arrows) were removed
240 and used for LC-MS/MS analysis to determine the proteins. Broad range (10-250kDa) protein
241 ladder was used on both gels; B) Cellular distribution of the proteins obtained in the pull-down
242 assay following LC-MS/MS analysis.
243

244 **In vitro analysis of the interaction of occidiofungin with purified actin.**

245 Typical assays for characterizing actin binding natural products are the *in vitro* F-actin

246 polymerization and depolymerization experiments. However, the addition of occidiofungin was

247 found to have no effect on the polymerization or depolymerization properties of F-actin (S5

248 Figure). Therefore, additional studies were required to confirm that actin was the biological target

249 for occidiofungin. Biotinylation of alkyne-OF following incubation with F- or G-actin and

250 streptavidin agarose beads was performed to determine whether occidiofungin directly associated

251 with purified actin *in vitro*. F- or G-actin incubated with the wild type occidiofungin or DMSO

252 were used as controls for potential non-specific interaction of actin with the agarose beads. The

253 eluant from the biotinylated alkyne-OF had a single band at approximately 42 kDa, the expected

254 size for actin. As shown in Fig 5A, the biotinylation of alkyne-OF was required for the co-

255 purification of F- or G- actin with the streptavidin beads (Lane 5 and 8) as actin was not present in

256 the control lanes that exposed actin to native OF or the carrier solvent DMSO (lanes 6, 7, 9, and

11

257   10). In this *in vitro* interaction assay, occidiofungin was shown to directly bind to F- or G-actin.

258   To further support this observation, a dissociation constant of 1.0 ± 0.8 μM was determined from

259   three independent ITC experiments using rabbit skeletal muscle G-actin (Fig 6). The ITC data also

260   showed a 1:1 binding ratio for occidiofungin to G-actin. The ITC experiments were not adaptable

261   to observe F-actin binding, so a co-sedimentation assay, which is commonly reported for

262   identifying actin associated proteins, was performed [51, 52]. Phalloidin was used as a positive

263   control in the assay (Fig 7). Phalloidin had an estimated dissociation constant (Kd) of 8 nM with

264   a saturation of binding ratio of 0.6 phalloidin to one actin monomer. These values are corroborated

265   by previous reports [53]. The biggest difference in binding to F-actin between occidiofungin and

266   phalloidin is the number of molecules bound before saturation. Phalloidin saturated at

267   approximately one molecule for every two actin monomers, whereas 24 molecules of

268   occidiofungin were bound to each actin monomer before saturation. Even with the large number

269   of bound occidiofungin to F-actin, the estimated Kd value was still 1.0 μM. It is important to note

270   that the higher Kd value is attributed to a 50-fold increase in the amount of occidiofungin bound

271   to actin compared to phalloidin.

272   Confocal microscopy using the fluorophore Acti-stain 670 phalloidin was carried out to

273   visualize the impact of occidiofungin or alkyne-OF on actin filaments. Microscopic evaluation

274   found that F-actin was still present but the morphology of F-actin changed in the presence of

275   occidiofungin in a dose-dependent manner (Fig 5B). Using alkyne-OF labeled with azide

276   functionalized Alexa Fluor 488 dye, occidiofungin interaction with F-actin was directly observed

277   (S6 Figure). Similar to that shown using labeled phalloidin, F-actin also appeared to aggregate

278   following exposure to occidiofungin. Fluorescence visualization of this interaction following

279   treatment with alkyne-OF and native occidiofungin demonstrated a high degree of aggregation of

12

280   the filaments which was not observed in the controls. Occidiofungin does not prevent

281   polymerization or depolymerization of F-actin, but it does bind to F-actin causing it to aggregate.

282   Given these unique observations for the bioactivity of occidiofungin, additional *in vivo* studies

283   were conducted to verify that actin is its biological target.

284   Fig 5. *In vitro* interaction of occidiofungin with F- and G-actin. a) Affinity pulldown of actin using
285   alkyne-OF: Lane 1- Ladder, Lane 2-100 ng pure F-actin, Lane 3-100 ng pure G-actin, Lane 4-
286   Empty, Lane 5-F-actin treated with alkyne-OF, Lane 6-F-actin treated with native occidiofungin,
287   Lane 7-F-actin treated with DMSO, Lane 8-G-actin treated with alkyne-OF, Lane 9-G-actin treated
288   with native occidiofungin, Lane 10-G-actin treated with DMSO; b) Fluorescence microscopy
289   analysis of the effect of occidiofungin treatment on actin filaments visualized using fluorescently
290   labeled phalloidin: A: actin filaments treated with solvent blank (DMSO), B: Actin:native
291   occidiofungin (24 μg actin:4 μg native occidiofungin), C: Actin:native occidiofungin (24 μg
292   actin:8 μg native occidiofungin). Scale bar represents 5μm.

293   Fig 6. Isothermal titration calorimetry is used to measure occidiofungin (OF) binding to actin. A)
294   Thermogram showing the heat exchange from equal injections of a solution containing OF into
295   the ITC chamber containing actin. B) A representative binding isotherm of the integrated heat
296   change from each injection shown in A is fit to a single-ligand binding model to yield an OF-actin
297   dissociation constant. The mean and standard deviation from three independent experiments are
298    $K_d = 1.0 \pm 0.8$ μM.

299   Fig 7. Co-sedimentation assay to demonstrate binding of occidiofungin to actin. A) Binding curve
300   of phalloidin to actin ($K_d = 8$ nM and the stoichiometry (ligand : protein) is 0.6:1.0) and B) Binding
301   curve of occidiofungin to actin ($K_d = 1$μM and the stoichiometry (ligand : protein) is 24:1). The
302   graph is plotted between amount of free occidiofungin obtained in the supernatant of the co-
303   sedimentation assay and the amount of bound occidiofungin obtained from the actin pellet. The
304   data was fit to a standard Langmuir binding isotherm of the form: $[X]bound = [X]*S/(K_d + [X])$,
305   where S is the maximal X bound, K_d is the dissociation constant and X is the concentration of free
306   ligand.

307

In vivo analysis of occidiofungin interactions with actin.

309   *In vivo* visualization of the localization of occidiofungin was done in intact yeast cells.

310   Cellular localization of F-actin is well characterized in *S. pombe* and *S. cerevisiae.* Time course

311   analysis of *S. pombe* following alkyne-OF treatment and derivatization with azide Alexa-488

312   showed a specific pattern of localization of the compound (Fig 8A). Alkyne-OF was seen to have

313   a faint pattern of staining at the polar tips at 10 minutes post treatment, which subsequently

314   increased in intensity at 30 minutes post treatment. Strong fluorescence was observed at the polar

315   ends of the cell and at the septum of dividing cells. A similar assay done using *S. cerevisiae* showed

316   localization of alkyne-OF at the bud tips at the early time points and staining throughout the parent

317   cell at later time points (Fig 8B). The unique pattern formed was observed to be a combination of

318   striated and inclusion-like structures. In both yeast systems, when cells were pre-treated with

319   native occidiofungin prior to treatment with alkyne-OF, the observed cellular localization patterns

320   disappeared (Fig 8 A & B, panels D, E, and F) indicating that alkyne-OF and occidiofungin

321   compete for the same cellular target. The vesicular pattern observed at the later time points of

322   exposure is indicative of endocytic vesicles that are coated with actin being circulated through the

323   cell [54, 55]. Actin patches in the cells of *S. pombe* are seen at the cell tips in growing cells and at

324   the division septum in dividing cells. Actin patches recruited to the division septum interact with

325   myosin to form the acto-myosin ring which is instrumental in cell division [56]. The time course

326   analysis in both types of fungal cells show localization of occidiofungin to the regions where actin

327   is known to be localized.

328   Fig 8. Competition assay of native occidiofungin and alkyne-OF. Time course analysis (A-C) of
329   alkyne-OF distribution and the distribution of alkyne-OF with the competition of native
330   occidiofungin (D-F) in a) *Schizosaccharomyces pombe* and b) *Saccharomyces cerevisiae*. Arrows
331   indicate specific localization patterns of alkyne-OF observed in each cell at 10, 30, and 60 minutes.
332   When pretreated with native occidiofungin, alkyne-OF does not bind or is restricted to cellular
333   envelope in *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*, respectively.

334   To directly determine the effect of occidiofungin on actin organization *in vivo*, fluorescence

335   microscopy was carried out on diploid cells of *S. cerevisiae* exposed to sub-inhibitory

336   concentrations of occidiofungin. Within 30 minutes of exposure, an accumulation of actin patches

337   and/or aggregates of F-actin were observed throughout the treated cells with a concomitant loss of

338 actin cables (Fig 9A and 9B). Actin cables are formed by bundling F-actin. In Figure 8, the

339 punctate structures in these cells are still likely filamentous actin, but occidiofungin appears to

340 disrupt the organization of F-actin to form cables at sub-inhibitory concentrations.

341 Fig 9. Effects of occidiofungin exposure on the integrity of actin cables in *S. cerevisiae* cells. A
342 montage of cells processed for actin visualization using phalloidin-TRITC from: a) a culture
343 exposed to solvent blank control (DMSO) where actin patches and cables are easily identifiable;
344 and b) an occidiofungin treated culture (0.5X MIC; for 30 minutes) showing loss of actin cables
345 and the accumulation of actin aggregates. Scale bars represent 2μm. The arrows are used to
346 demarcate the presence of actin cables.

347

348 **Efficacy of occidiofungin in treating a murine model of vulvovaginal candidiasis.**

349 Six to eight-week-old BALB/c mice that were intravaginally infected with *C. albicans* were

350 dosed once per day with occidiofungin for three days. The occidiofungin treated groups were

351 compared to a vehicle control group. Three groups of six mice were treated with 100, 50, and 0 μg

352 of occidiofungin suspended in 0.3% Noble agar. The occidiofungin treated groups reduced fungal

353 load by more than two logs (Fig 10). The reduction in fungal load with both treatment groups was

354 statistically significant from vehicle control ($p<0.001$). There was no statistically significant

355 difference between the treated groups ($p=0.33$), suggesting that the lower limit of occidiofungin

356 dosing was not achieved in the experiment. During the course of the study, the mice were examined

357 for outward signs of distress or irritation. No behavioral changes including sluggishness,

358 stretching, or reluctance to consume food was observed. Furthermore, no vaginal bleeding or

359 swelling was observed following treatment.

360 Fig 10. Efficacy of occidiofungin in treating murine vulvovaginal candidiasis. The graph
361 demonstrates CFUs per ml of *Candida albicans* in the control group of mice compared to the
362 groups treated intravaginally with different concentrations of occidiofungin in 0.3% noble agar.
363 Error bars represent standard deviation. Statistical analyses indicate a significant difference
364 between the control group and the treated groups ($p<0.001$) as indicated by the asterisk.

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Discussion

367   We have demonstrated that occidiofungin directly interacts with actin causing a disruption

368   in normal cellular actin-based functions. *In vivo* data in yeast indicates that following treatment,

369   the compound accumulates in areas rich in actin localization. In addition, disruption of cables

370   throughout the fungal cells following exposure to sub-inhibitory concentrations of occidiofungin

371   can be visualized. This interaction with actin can be observed *in vitro* via pulldown assays, ITC

372   assays, and co-sedimentation studies. These studies establish direct interaction of occidiofungin

373   with actin with a dissociation constant similar to other well-established actin binding proteins such

374   as α-actinin, tropomyosin isoforms, and fimbrin [57-59]. From the binding studies and *in vitro*

375   microscopy assays, it is evident that occidiofungin likely assembles into a large complex around

376   F-actin. Based on the ITC study, occidiofungin and G-actin were bound at a 1:1 ratio. The

377   propensity to form an occidiofungin complex appears to require the presence of F-actin. Formation

378   of self-assembled complexes has previously been observed in several lipopeptide antibiotics [60].

379   It is likely that occidiofungin undergoes self-assembly, forming micellar structures at the

380   concentrations tested in our assay. Additionally, cellular processes that rely on the maintenance of

381   the actin cytoskeleton such as endocytosis, hyphae formation, and nuclear DNA positioning were

382   shown to be disrupted with the addition of occidiofungin. We also demonstrate that occidiofungin

383   is capable of treating a murine vulvovaginal candidiasis infection without any signs of toxicity.

384   Furthermore, occidiofungin demonstrated efficacy at a concentration that is ten-fold lower than

385   azole-based treatment methods [61].

386   Given that we have observed fungicidal activity as low as 100 nM concentrations, the

387   number of bound occidiofungin to F-actin is likely to be a lot lower than 24:1 for its fungicidal

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388   activity. We hypothesize that occidiofungin binding to F-actin interferes with the binding of other

389   actin associated proteins leading to disruption of cellular activities involving actin dynamics.

390   Cables are necessary for a multitude of cellular functions including hyphal formation (disrupted

391   following treatment as seen in Figure 2), endocytosis (reduced following treatment as seen in

392   Figure 3) and proper positioning of the mitotic spindle during cell division (accumulation of

393   multinucleated cells following treatment as seen in S2 Table). Studies aimed at understanding the

394   events following the binding of occidiofungin to actin will need to be conducted to determine the

395   exact connection for inducing apoptosis. Further, the localization pattern observed in the

396   microscopy studies done in *S. cerevisiae* and *S. pombe* demonstrate the specificity of occidiofungin

397   to F-actin. If the binding to actin were non-specific, actin would act as a retardant to the efficacy

398   of occidiofungin due to the sheer number of occidiofungin molecules that are capable of binding

399   a single F-actin monomer at a Kd value in the range of most actin associated proteins. The co-

400   sedimentation assays and the localization studies indicate that the primary target of occidiofungin

401   in the yeast cell is actin.

402   One of the challenges facing the development of antifungals is the fact that uptake of

403   compounds into fungal cells does not occur as easily as it does in bacteria. Most fungal cells have

404   a sturdy cell wall made of several glycoproteins that make up almost one-third of the dry weight

405   of the cell. The efficiency of antifungals relies heavily upon being able to penetrate the cell

406   envelope [62]. Occidiofungin has the advantage of being taken up by the fungal cell, as evidenced

407   by the low MICs against several different types of fungi. Susceptibility to occidiofungin can be

408   seen in pathogenic strains that are resistant to treatment with azoles and echinocandins (S1 Table).

409   Additionally, occidiofungin has the advantage of inducing cell death in fungi via a mechanism that

410   differs from the common classes of antifungals used to treat fungal infections.

411   Recent studies have shown that the dynamic nature of actin is necessary to maintain the

412   cellular functions in which actin is involved such as endocytosis, mitochondrial transport, and

413   growth [45]. A disturbance in the actin dynamics affects mitochondrial function [63, 64]. Previous

414   reports suggest that stabilization and aggregation of actin leads to the induction of a Ras-cAMP-

415   PKA pathway which causes mitochondrial destabilization and production of ROS [65, 66]. We

416   have demonstrated that occidiofungin can directly interact with actin. Loss of actin cables

417   following occidiofungin treatment can affect mitochondrial integrity, which in turn triggers a

418   cascade of events leading to the release of reactive oxygen species. The release of ROS has been

419   widely reported to be a precursor for the onset of apoptosis in yeast [67]. In addition, caspase

420   dependent pathways have been theorized to be induced following aggregation of actin filaments

421   in animal cells and it is possible that a similar pathway takes place involving Yca1p, the caspase

422   found in yeast [68]. A newly formed bud contains a large pool of actin which coordinate the

423   retrograde transport of vesicles along the actin cable into the mother cell. Actin nucleation is

424   carried out by the Arp2/3 complex and a host of proteins including Cap1, Abp1 and Sac6 which

425   are involved in the actin patch based transport of vesicles [45]. Fluorescence time course assays

426   done on the cells of *S. cerevisiae* and *S. pombe* support this hypothesis. Furthermore, bud tips in

427   *S. cerevisiae* are known to be rich in actin patches which are necessary to carry out cellular

428   functions such as cell division and endocytosis [45]. Occidiofungin was observed to localize to

429   these cellular areas in the *in vivo* microscopy experiments.

430   Natural products with *in vitro* antifungal properties that target the actin cytoskeleton have

431   been previously reported [69]. One of the examples of an actin targeting antifungal is

432   jasplakinolide, a compound that was isolated from sea sponges[70]. Although the compound had

433   comparable activity against some *Candida* species when compared with miconazole, it had a

434   limited spectrum of activity [70]. Furthermore, jasplakinolide was reported to be severely toxic in

435   animal systems. It was reported to cause necrosis at the site of a subcutaneous injection at doses

436   as low as 0.1 mg/kg and led to mortality at 1 mg/kg [71]. It is possible that its high level of toxicity

437   is associated to the direct inhibition of actin depolymerization or its interaction with other cellular

438   targets. A newly discovered actin binding antifungal is ginkbilobin [72]. This protein has been

439   reported to induce programmed cell death following perturbation of the actin cytoskeleton and has

440   a broader range of activity compared to jasplakinolide, however the effect of the compound in an

441   animal system has not been reported [73]. Similarly, neosiphoniamolide A, a cyclodepsipeptide

442   closely related to jasplakinolide, demonstrated a wide spectrum of antifungal activity. However,

443   its toxicity in an animal system has not yet been reported [74]. Halichondramide, another

444   antifungal compound isolated from a marine sponge, has been reported to be active against

445   *Candida* species and *Trichophyton* species. Like jasplakinolide, it was severely toxic following a

446   subcutaneous administration of 1.4 mg/kg of the compound in a murine system [75]. To date, the

447   short list of actin binding antifungal compounds is limited by their toxicity in animals at doses that

448   would be required to demonstrate an efficacious effect.

449   Occidiofungin, on the other hand, is well tolerated at 5 mg/kg or 20 mg/kg when

450   administered intravenously or subcutaneously, respectively[37, 40]. This dose is much higher than

451   the MIC of occidiofungin against several fungal pathogens. Furthermore, blood cells and blood

452   chemistry following administration of occidiofungin show no serious signs of toxicity [40]. The

453   difference in reported toxicity of occidiofungin to other actin binding compounds may be attributed

454   to the inability of occidiofungin to disrupt polymerization or depolymerization of actin, different

455   rates of uptake between yeast and animal cells, or the possible lack of off target binding that could

456   lead to a toxic response. We have previously shown that occidiofungin is effective against several

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457   cancer cell lines [40]. The activity against these cell lines was almost a log more sensitive than the

458   fibroblast cell line used as a control. It is believed that the growth rates of the cells are attributed

459   to the differences in killing activity. This was later demonstrated with *S. cerevisiae, C. albicans*

460   *and C. glabrata* strains of yeast grown in nutrient depleted conditions [49]. Further studies need

461   to be carried out to examine the lack of severe toxicity of occidiofungin to an animal system. The

462   low toxicity of occidiofungin combined with its wide spectrum of activity and demonstrable *in*

463   *vivo* efficacy in treating a fungal infection is unprecedented in any actin binding antifungal

464   compound.

465   Occidiofungin binds to actin and causes aggregation of the F-actin filaments *in vitro* which

466   may lead to the accumulation of ROS and apoptosis *in vivo*. Demonstration of the effect of the

467   compound in eliminating a common fungal infection *in vivo* supports the belief that occidiofungin

468   could constitute a new class of clinically relevant antifungals. The activity of occidiofungin against

469   echinocandin and azole resistant strains of pathogenic fungi leads us to consider that this

470   compound would be a valuable addition to the existing antifungals for clinical treatment.

471   Additional studies with occidiofungin may aid in furthering the understanding of the cellular

472   events taking place that lead to the accumulation of ROS and apoptosis. A better understanding of

473   entry and the events that lead to apoptosis following the binding of actin may lead to other

474   potentially novel therapeutics. Further, future studies aimed at understanding how occidiofungin

475   crosses the plasma membrane of fungi are warranted. Nevertheless, an actin-targeting antifungal

476   that has a wide spectrum of activity against clinically pathogenic fungi and minimal toxicity in

477   animal models could be the novel drug that is needed in the current antifungal arsenal to combat

478   fungal infections.

479

Methods

Spectrum of activity of occidiofungin.

Minimum inhibitory concentration (MIC) susceptibility testing was performed according to the CLSI M27-A3 and M38-A2 standards for the susceptibility testing of yeasts and filamentous fungi, respectively. Incubation temperature was 35˚C and the inoculum size was $0.5 – 2.5 \times 10^3$ colony-forming units (CFU)/mL and $0.4 - 5 \times 10^4$ conidia/mL for yeasts and filamentous fungi, respectively. Inoculum concentration for dermatophytes was $1-3 \times 10^3$ conidia/mL. RPMI was used throughout as the growth medium and *Cryptococcus* strains were tested in YNB. Occidiofungin MICs were recorded at 50% and 100% growth inhibition after 24 and 48 hours of incubation, with the exception of dermatophytes which were incubated for 96 hours. Fluconazole MICs against *Candida* strains were recorded at 50% inhibition after 24 hours and against *Cryptococcus* strains after 72 hours. Voriconazole MICs were recorded at 100% inhibition after 24 hours for zygomycetes and after 48 hours for *Fusarium* and *Aspergillus* strains. Voriconazole MICs were recorded at 80% inhibition after 96 hours of incubation for dermatophytes. *S. cerevisiae* deletion mutants were obtained from the commercially available BY4741 deletion library (Thermo Scientific). Susceptibility testing was carried out on inoculum size of $0.5-1 \times 10^4$ cells/ml in YPD media at 30˚C and MICs recorded after 48 and 60 hours.

Hyphal induction.

Candida albicans strain ATCC 66027 was grown in YPD at 30˚C for 48 hours to reach saturation with a density of 17-19 OD_{600}/ml. Cells were diluted into fresh Spider media (1% nutrient broth, 1% mannitol, 0.2% K_2HPO_4) to obtain 0.05 OD_{600}/ml (~$0.5-1 \times 10^6$ cells/ml) and incubated at 37˚C with shaking to induce hyphae formation[76]. Immediately prior to 37˚C incubation, occidiofungin (1μg/ml; 0.5X MIC) or an equal volume of DMSO was added to

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503   cultures. Aliquots were removed at 0, 1, 2, 4, and 6 hour time points, fixed in 3.7% formaldehyde

504   for later analysis of cell morphology by light microscopy. Over 200 cells were examined for each

505   time point and cells were scored as having either a yeast or filamentous form with cells showing

506   any outgrowth as being filamentous.

507   **DNA segregation.**

508   A mid log culture of *S. cerevisiae* (BY4743; diploid) and *C. albicans* (ATCC 66027) were

509   diluted into fresh YPD to obtain an OD_{600}/ml of 0.095 (~1-1.5x10^6 cells/ml) and 0.05 (0.5-0.8x10^6

510   cells/ml), respectively. DMSO or occidiofungin (1µg/ml; 0.5X MIC) was added and cultures were

511   placed at 30˚C with shaking. Samples were removed at 0.5, 1, and 2 hours and cells fixed for 1.5

512   hours at room temperature with the addition of formaldehyde to 3.7%. Cells were washed in PBS

513   and permeabilized with the addition of an equal volume of PBS containing 0.2% TritonX-100 for

514   30 minutes at room temperature. Cells were isolated by centrifugation, washed in PBS,

515   resuspended in a minimal volume of PBS, added to concanavalinA treated glass slide, and overlaid

516   with VectaShield plus DAPI. Images were viewed using a Nikon 50i fluorescence microscope with

517   a 100X oil immersion objective and DAPI filter. Cells were scored based on bud morphology and

518   DNA location.

519   **FM-464 uptake assay for endocytosis.**

520   Three 1 mL aliquots of *S. pombe* at a density of OD_{600} 0.6 to 0.8 were treated with 0.01%

521   DMSO, 0.5 µg/mL (0.5X MIC), or 1 µg/mL (1X MIC) of native occidiofungin for 30 minutes at

522   30˚C. Cells were isolated by centrifugation at 21,000 g for 2 minutes, washed thrice with PBS and

523   resuspended in YPD containing 8 mM FM-464 (ThermoFisher Scientific). Cells were incubated

524   in the presence of the dye for 60 minutes at 30˚C, followed by two washes with PBS and then

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525   added to a microscope slide for visualization. Images were obtained using an Olympus FV1000

526   confocal microscope with a 40x/0.9 dry objective.

527   **Derivatization of occidiofungin.**

528   Occidiofungin was purified from a liquid culture of *Burkholderia contaminans* MS14 as

529   previously described [36]. Pure occidiofungin was aliquoted into 100 μg fractions and stored in

530   lyophilized form at 4˚C until use. Addition of an alkyne reactive group to the primary amine on

531   occidiofungin was performed initially at the Texas A&M Natural Products LINCHPIN Laboratory

532   at Texas A&M University and subsequently at the CPRIT Synthesis and Drug-Lead Discovery

533   Laboratory at Baylor University.

534   The alkyne derivatization of occidiofungin was carried out under nitrogen atmosphere in a

535   round bottom flask (S1 Figure) using HPLC grade reagents. A solution of triethylamine and

536   acetonitrile was prepared by dissolving 10 μL of trethylamine in 11.7 mL of acetonitrile. A stock

537   solution of acetonitrile and reagents was prepared by adding 2,5-dioxopyrrolidin-1-yl hex-5-

538   ynoate (1.40 mg, 6.69 μmole, 8.2 equiv.) to 1.08 mL of the triethylamine and acetonitrile solution

539   prepared above. Occidiofungin (1 mg, 0.82 μmole, 1 equiv.) was added to the stock solution of

540   acetonitrile and reagents (400 μL) containing triethylamine (0.34 μL, 2.46 μmole, 3 equiv.) and

541   2,5-dioxopyrrolidin-1-yl hex-5-ynoate (51.6 μg, 2.46 μmole, 3 equiv.). Occidiofungin was added

542   while stirring, with water (400 μL) added to the mixture as a co-solvent. The resultant mixture was

543   stirred for three days at 22˚C. Purification of alkyne-OF was performed on an Agilent 1200 series

544   semi-prep HPLC (gradient 5% acetonitrile/water to 95% acetonitrile/water over 20 minutes) using

545   a Phenomenex Gemini 5μ C18 110A (100 x 21.2 mm) reversed-phase column. For analytical

546   analysis, a phenomenex Gemini 3μ C18 110A (150 x 4.6 mm) reversed-phase column was used

547   on an Agilent 1200 series analytical HPLC system. Derivatized occidiofungin was also purified

548   using a 4.6- by 250-mm C18 column (Grace-Vydac; catalog no. 201TP54) on a Bio-Rad BioLogic

549   F10 Duo Flow with Quad Tec UV-Vis detector system using a similar gradient described above.

550   Analytical HPLC analysis of the reaction mixture indicated complete consumption of the starting

551   material and a new peak in the chromatogram (OF RT = 13.6 min; alkyne-OF RT = 15.0 min).

552   The crude mixture was lyophilized and the crude powder, solubilized in 35% acetonitrile

553   containing 0.1% trifluoracetic acid, was purified on a semi-prep HPLC (alkyne-OF RT = 13.0

554   minutes) column to yield alkyne-OF (0.76 mg, 70%).

555   NMR data were collected using a Bruker Ascend 600 (^{1}H 600 MHz and ^{13}C 150 MHz)

556   NMR spectrometer equipped with a 5 mm cryoprobe. The NMR data for ^{1}H NMR chemical shifts

557   are reported as δ values in ppm relative to residual HOD (3.30 ppm), coupling constants (J) are

558   reported in Hertz (Hz), and multiplicity follows convention. Unless otherwise indicated,

559   dimethylsulfoxide-d6 (DMSO-d6) served as an internal standard (40.5 ppm) for the ^{13}C spectra.

560   NMR data for alkyne-OF A-D is shown in S2 Figure (A-F) and the assignment of the alkyne

561   subunit to diaminobutyric acid (DABA5) is shown in S3 Figure (A-H). The assignment of the

562   alkyne subunit has led to a correction in the previously reported configuration of DABA5[28] and

563   occidiofungin B is likely identical in structure to Bk-1215[50] isolated by Schmidt et al.

564   Assignment of the High-resolution mass spectra (HRMS) were run on a Thermo LTQ Orbitrap

565   mass spectrometer with ESI direct infusion (alkyne-occidiofungin B HRMS (ESI$^+$): Calcd. For

566     $C_{58}H_{92}N_{11}O_{23}$ ([M+H]$^+$), 1310.6360. Found: 1310.6361).

567   **Actin polymerization and depolymerization assays.**

568   The effect of unmodified occidiofungin on actin polymerization and depolymerization was

569   measured using the Actin Polymerization Biochem Kit (fluorescence format): rabbit skeletal

570   muscle actin from Cytoskeleton Inc. (Catalog no.: BK003). Occidiofungin was brought up in 1.5%

571   β-cyclodextrin in PBS (pH 7.5) at a concentration of 1 μg/μL and 20 μL of this solution was used

572   per well. The same buffer without occidiofungin was used for buffer control. General Actin buffer

573   was supplemented with ATP to 0.2mM prior to the start of the experiment as instructed. G-actin

574   and F-actin stocks were prepared as described in the kit to achieve stock concentrations of 0.4

575   mg/mL and 1 mg/mL, respectively. Polymerization and depolymerization assays were then carried

576   out as per the manufacturer's instructions.

Alkyne-OF functionality testing.

578   Stock solutions of unmodified occidiofungin and alkyne derivatized occidiofungin were

579   made in DMSO at a concentration of 1mg/mL. These stock solutions were utilized in all the assays

580   described in this manuscript. The activity of the purified alkyne-OF was compared to the native

581   compound using the CLSI M27-A3 method of determination of the minimum inhibitory

582   concentration (MIC) against *Saccharomyces cerevisiae* BY4741 and *Schizosaccharomyces pombe*

583   972h (received from Dr. Susan Forsburg, Department of Biological Sciences, University of

584   Southern California). Additionally, activity of the alkyne derivatized occidiofungin was tested

585   against a higher density (OD$_{600}$/mL 0.6 to 0.8) of cells for both yeast. Assays for TUNEL (APO™-

586   BrdU TUNEL Assay Kit, LifeTechnologies), phosphatidylserine externalization (Annexin-V-

587   Fluos staining kit, Roche), and ROS detection (Dihydrorhodamine 123, Sigma) were carried out

588   as previously described[39].

Affinity purification of alkyne-OF associated proteins.

590   One mL of cells from an overnight culture of *S. pombe* grown to an OD$_{600}$ of 0.6-0.8. was

591   incubated with 8 μg/mL of alkyne-OF for 30 minutes at 30˚C. Controls included cells treated with

592   DMSO and native occidiofungin. An additional sample using cells that were lysed prior to alkyne-

593   OF treatment was used for comparison (Figure 2A, Lane Post Click). Following exposure, cells

594   were isolated by centrifugation, washed once with PBS and then lysed by probe sonication (30

595   second sonication followed by 30 second on ice; cycle repeated 5 times). Insoluble material was

596   removed by centrifugation at 16,000 g for 10 minutes and the supernatant used in a Click-it protein

597   reaction (Life Technologies) with the alkyne-OF reacted with azide-biotin, as per the

598   manufacturer's instructions. The reaction was allowed to proceed for 90 minutes at 37˚C with

599   shaking. Unreacted reagents were removed by passage through a Microcon 10 kDa cutoff filter

600   (Sigma Aldrich) and the concentrated proteins solubilized in 100 μL of 100 mM Tris HCl (pH

601   7.5). Biotinlyated proteins were selected using streptavidin agarose beads (ThermoFisher

602   Scientific) with incubation at 37˚C for 90 minutes. Beads were washed with 10 mL of 100 mM

603   Tris HCl (pH 7.5) and bound proteins extracted with boiling in 50 μL of 1X SDS sample loading

604   buffer for 15 minutes. The resulting protein sample was loaded onto a 12% SDS gel and

605   electrophoresis was carried out until the bromophenol blue dye was ~1cm into the separating gel.

606   The resulting band was excised, trypsin digested by the Protein Chemistry Laboratory (Texas

607   A&M University), and the subsequent LC-MS/MS analysis performed by the Mass Spectrometry

608   Laboratory at the University of Texas Health Science Center (San Antonio). Results were analyzed

609   using the Scaffold software.

610   **Intracellular localization of alkyne-OF.**

611   Colonies from a freshly streaked plate were used to inoculate an overnight culture of *S.*

612   *pombe* or *S. cerevisiae.* 1 mL of cells from a culture with an OD_{600} of 0.6 to 0.8 was incubated

613   with MIC quantities of alkyne-OF at 30˚C with 200 μL samples removed at 10, 30, and 60 minute

614   post incubation. Cells were isolated by centrifugation, washed once in phosphate buffered saline

615   (PBS), and fixed for 15 minutes at room temperature with the addition of formaldehyde to 3.7%

616   (in PBS). Cells were permeabilized at room temperature for 20 minutes with the addition of 0.5%

617   TritonX-100 (in PBS). Cells were washed twice with 1 mL of PBS each. Click reaction with azide

618   derivatized Alexa-488 was carried out according to the manufacturer's protocol (Click-iT EdU

619   Imaging kit, ThermoFisher Scientific). Cells were washed with PBS and added to a microscope

620   slide for visualization using an Olympus FV1000 confocal microscope with a 100x/1.4 oil

621   immersion objective and 40x/0.9 dry objective. A competition assay was carried out by pre-

622   treating cells with an MIC amount (0.5 μg/mL) of the native occidiofungin followed by treatment

623   with alkyne-OF.

In vitro actin binding.

625   Purified rabbit skeletal muscle filamentous actin (Catalog: AKF99) and G-actin (Catalog:

626   AKL95) was purchased from Cytoskeleton Inc. Actin was reconstituted in Milli-Q water to

627   achieve a stock concentration of 0.4 mg/mL in a buffer that consisted of 5 mM Tris-HCl (pH 8.0),

628   0.2 mM $CaCl_2$, 0.2 mM ATP, 2 mM $MgCl_2$, and 5% (w/v) sucrose as directed by the supplier. This

629   solution was stored at -80°C in 50 μL aliquots until use. Immediately before use, each aliquot was

630   thawed by placing in a 37°C water bath for 5 minutes followed by room temperature. For all

631   studies, 24 μg of F- or G-actin was used with 8 μg alkyne-OF.

632   For biochemical-based studies, click chemistry was performed on this mixture to react the

633   alkyne-OF with azide-biotin for 90 minutes as described (Click-iT protein reaction buffer kit,

634   ThermoFisher Scientific). Unreacted reagents were removed by centrifugation (20 minutes at

635   15,000x g) through a 10 kDa cutoff filter. Proteins retained in the filter chamber were solubilized

636   in 200 μL of 100 mM Tris-HCl (pH 7.5) and reacted with streptavidin beads as described above.

637   The beads were washed multiple times using 100 mM Tris HCl (pH 7.5) and bound proteins eluted

638   by boiling in 50 μl of SDS sample loading buffer. The sample was electrophoresed through a 12%

639   SDS gel and protein bands visualized by silver staining according to the manufacturer's protocol

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640   (Pierce Silver stain kit, ThermoFisher Scientific). F- and G-Actin treated with DMSO and native

641   occidiofungin were used as controls.

642   For microscopy-based studies, click chemistry was performed to react the alkyne-OF with

643   functionalized Alexa Fluor 488 according to the manufacturer's instructions (Click-iT EdU

644   Imaging kit, ThermoFisher Scientific). Unbound dye was removed by overnight dialysis at 4˚C

645   against actin polymerization buffer using a 1 kDa cutoff membrane (Catalog no.: BSA02,

646   Cytoskeleton Inc.). Actin filaments were removed, added to a slide and analyzed using an Olympus

647   FV1000 confocal microscope 40x/0.90 dry objective and a 100x/1.4 oil immersion objective. As

648   a control, 140 nM Acti-stain 670 phalloidin (Cytoskeleton Inc.) was used to visualize actin

649   filaments as per manufacturer's instructions.

650   F-actin filaments were reacted with native occidiofungin for 15 minutes at room

651   temperature at molar ratios of 1:10 (24 μg actin:8 μg native occidiofungin) and 1:5 (24 μg actin:4

652   μg native occidiofungin). The respective mixtures were then stained with 140 nM Acti-stain 670

653   phalloidin for an additional 15 minutes at room temperature. Stained filaments were added to a

654   glass slide and observed on an Olympus FV1000 confocal microscope using a 100x/1.4 oil

655   immersion objective. Actin filaments treated with DMSO (solvent blank negative control) were

656   stained and observed for comparison.

657   **Isothermal Titration Calorimetry**.

658   Rabbit skeletal muscle actin (Cytoskeleton, Inc.) was re-constituted as described above.

659   Aliquots of actin were thawed and incubated for 1 hour in ITC buffer which contained General

660   Actin Buffer (Cytoskeleton, Inc.: 5 mM Tris-HCl, pH 8.0, 0.2 mM $CaCl_2$) plus 0.5 mM DTT, 0.2

661   mM ATP and 5% DMSO. Lyophilized OF was re-suspended in ITC buffer to yield a 300μM

662   concentration. The ITC chamber was loaded with 30 μM G-actin and the syringe with 300 μM OF.

663 A MicroCal iTC200 (Malvern Instruments, Spectris plc) was used to obtain OF binding to actin at

664 25˚C. A 0.3 µL injection was followed by 13 injections of 3 µL while stirring at 1,000 rpm. The

665 integrated heat from each injection was fit to a one-site binding model using Origin.

666 Co-sedimentation studies.

667 Reaction buffer for F-actin binding study consisted of 5 mM Tris-HCl (pH 8.0), 0.2 mM

668 ATP, and 2 mM $MgCl_2$. Samples were prepared in 100 µL volume and the final concentration of

669 F-actin was 200 nM. Occidiofungin was added to the reaction at concentrations from 25 to 25600

670 nM. Phalloidin was added to the reaction at concentrations from 25 to 800 nM. Samples were

671 incubated at room temperature for 30 minutes in thick-wall polycarbonate tubes (#349622,

672 Beckman Coulter, CA). After incubation, samples were centrifuged at 100,000 rpm, 4˚C, for 20

673 minutes to pellet F-actin (Beckman TL-100 ultracentrifuge). Both supernatant and pellet were

674 extracted with 200 µL solvent consisting of a combination of 70% acetonitrile (ACN) containing

675 0.1% trifluoroacetic acid (TFA) and 30% methanol containing 0.4% formic acid. The extracted

676 sample was centrifuged at 15,000 g for 10 minutes, after which the supernatant was transferred to

677 a clean centrifuge tube before being freeze dried in a SpeedVac (Labconco, Cat#7810010). The

678 dried sample was brought up to 100 µL with 50% ACN with 0.1%TFA containing 1 µg/mL

679 concentration of the internal standard and analyzed on LC-MS. An analog of occidiofungin served

680 as the internal standard of native occidiofungin, while native occidiofungin served as the internal

681 standard of phalloidin. All experiments were done in duplicate. An Agilent 1200 front end

682 chromatography system and a TSQ Quantum™ Access Triple Quadrupole Mass Spectrometer

683 were used to analyze the samples. Following a 10 µL injection, samples from binding study with

684 occidiofungin were separated using a 15-minute water/ACN (containing 0.2% formic acid)

685 gradient starting from 95% to 40% water on a C18 column (SinoChrom ODS-BP 5µm, 2.1 mm x

29

686   50 mm). The mass spectrometer was operated in positive mode and operated using a protocol

687   optimized for phalloidin and occidiofungin. Briefly, occidiofungin was monitored in SRM mode

688   with a scan width (m/z) of 0.3 and a collision energy of 31 eV, while phalloidin was monitored in

689   SIM mode with a scan width of 0.7. The parent and product mass of native occidiofungin were

690   1216.7 and 1084.7 Da, respectively. The center mass of phalloidin was 789.87 Da. Area of each

691   compound was measured through manual integration using Xcalibur™ Software (Thermo Fisher

692   Scientific). The standard curves were generated for each compound following the extraction

693   procedure described above. The R^2 values for each standard curve exceeded 0.99.

694   **Rhodamine-Phalloidin staining of actin *in vivo*.**

695   A mid log culture of *S. cerevisiae* (diploid; BY4743) was diluted into fresh YPD to obtain

696   0.095 OD_{600}/ml (~1-1.5x10^6 cells/ml) and occidiofungin (1μg/ml; 0.5X MIC) or an equal volume

697   of DMSO was added. Cultures were placed at 30˚C with shaking for 0.5 or 2 hours. Formaldehyde

698   (3.7% final) was added directly to the culture and cells incubated for 1.5 hours at room

699   temperature. Formaldehyde was removed by vacuum filtration through 0.2μm nitrocellulose

700   followed by several PBS washes. Cells were resuspended in PBS and permeabilized with the

701   addition of an equal volume of 0.2% Triton X-100 PBS solution.   Phalloidin-

702   Tetramethylrhodamine B isothiocyanate (3.3mM in 100% DMSO; Sigma) was added to 6.6μM

703   and cells stained for 30 minutes at room temperature. Unbound phalloidin-TRITC was removed

704   by centrifugation at 8,000 *g* for 8 minutes at 20˚C, the cells resuspended in PBS and added to

705   concanavalinA treated glass slide and overlaid with VectaShield plus DAPI. Images were viewed

706   using a Nikon 50i fluorescence microscope with a 100x oil immersion objective and Texas-Red

707   and DAPI filter sets. Random images were captured using a Retiga EXi Black and White CCD

30

708   Camera and Image Q software. All images were captured using the same exposure settings with

709   image contrast altered post capture using CorelDraw.

710   **Efficacy studies using occidiofungin.**

711   The murine model of vulvovaginal candidiasis has been widely reported[77]. A variation

712   of the method described was followed. Three groups of six mice were used to evaluate two

713   concentrations of occidiofungin (100 μg and 50 μg) and vehicle control (0.3% Noble agar). Briefly,

714   six to eight-week-old BALB/c mice were treated subcutaneously with 200 ng per mouse of β-

715   Estradiol 17-valerate three days prior to inoculation with *C. albicans* (D-3). A subcutaneous dose

716   of estradiol was administered every three days (D0, D3) until the end of the experiment to induce

717   pseudo-estrus. Approximately a 20 μL intravaginal inoculation of a 2.5×10^6 colony forming units

718   (CFU)/mL of *C. albicans* defines day zero (D0) of the VVC study. On the same day of inoculation

719   (D0), another subcutaneous injection of estradiol was made. Lyophilized powder of occidiofungin

720   containing either 100 μg or 50 μg of occidiofungin was suspended in 20 μL of warm 0.3% Noble

721   agar before intravaginal inoculation. Drug treatment was done on day 2 (D2), day 3 (D3) and day

722   4 (D4) of the study. On day 5 (D5), the vaginal lumen was lavaged with 100 μL of sterile PBS

723   with a 200 μL pipette tip. Serial dilutions and total colony forming units per vaginal lavage were

724   determined by plating on YPD plates containing 50 μg/mL of chloramphenicol. The colony

725   forming units (CFUs) obtained from each lavage were counted on plates containing 30-300

726   colonies for determining the CFU/mL estimates. Body weight, signs of vaginal irritation such as

727   swelling or bleeding and clinical signs of discomfort (stereotypical stretching behavior) were

728   monitored. Statistical analyses (T-test) were done to compare the control group to treated groups

729   and to compare differences between treated groups. All the analyses were 2-sided, with $P < .05$

730   considered statistically significant.

731 **Ethics Statement.**

732   Research Compliance's Animal Welfare Office (AWO) supports Texas A&M's

733 Institutional Animal Care and Use Committees (IACUC), through which all faculty, staff, and

734 students using animals, regardless of location or funding, must obtain approval before activities

735 begin. The committee approved the study titled "Determination of efficacy of occidiofungin in the

736 treatment of vulvo-vaginal candidiasis (IACUC number: IACUC 2017-0164)". The specific

737 national guidelines followed by Texas A&M's AAALAC accredited animal facilities are the

738 USDA animal welfare assurance regulations (Texas A&M registration: 74-R0012) and PHS NIH

739 Guidelines (Texas A&M registration: A3893-01).

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Supporting Information

S1 Table: Activity of occidiofungin against filamentous and non-filamentous fungi.

S2 Table: Occidiofungin exposure results in nuclear segregation defects in mitotic cultures of *S. cerevisiae* and *C. albicans*. Nuclear DNA was scored by DAPI staining of fixed cells treated with 0.5X MIC occidiofungin for 0.5, 1, and 2 hours at 30˚C. Cells were binned into one of four categories based on bud morphology and DNA localization and the percentage of cells in each category are reported. Approximately 200 cells were scored for each time point and data from two separate experiments are shown.

S3 Table: Activity of occidiofungin against *S. cerevisiae* mutants deleted for genes linked to actin polymerization and depolymerization.

S4 Table: Activity of alkyne-OF compared to native occidiofungin

S5 Table: List of proteins pulled down exclusively by alkyne-OF using the affinity purification. Proteins in the cells highlighted in green are those that were found in the pulldown assays in both *S.pombe* and *S.cerevisiae*. Proteins in the cells that are not highlighted were found in the *S.pombe* assays only.

S1 Figure: Scheme of chemical addition of alkyne group to occidiofungin B and mass determination of alkyne-OF B.

S2 Figure: NMR data for Alkyne-occidiofungin A-D (^{1}H 600 MHz and ^{13}C 150 MHz in DMSO-d6) A) ^{1}H NMR, B) COSY 2D NMR, C) TOCSY 2D NMR, D) HSQC 2D NMR, E) HMBC 2D NMR, and F) NOESY 2D NMR.

S3 Figure: Assignment of alkyne subunit and revised structure of diaminobutyric acid in the cyclic peptide. A) Expanded 1H NMR, B) Expanded TOCSY, C) Expanded HSQC, D) Expanded HMBC, E) Further expansion of HMBC, F) Expanded COSY NMR, G) Expanded TOCSY NMR, and H) Carbon and proton assignments of alkyne subunit.

S4 Figure: Induction of apoptosis by alkyne-OF: The 'DMSO' and 'H$_2$O$_2$' columns represent the negative and positive controls, respectively. The 'WT' column corresponds to cells treated with 1x MIC quantity of native occidiofungin and the last two panels represent treatment of cells with alkyne-OF at the concentration indicated. A) Externalization of phosphatidylserine demonstrated by the fluorescence of Annexin-V-Fluorescein, B) Release of reactive oxygen species indicated by the formation of rhodamine from dihydrorhodamine 123 and C) Double stranded breaks visualized by TUNEL assay, following treatment with native and alkyne-OF.

968 S5 Figure: Effect of occidiofungin on actin (a) polymerization and (b) depolymerization *in vitro*.

969 Symbols are as follows: ♦- G-buffer (control), ■ - G-buffer and pyrene actin, ▲ - Test buffer

970 (1.5% β-cyclodextrin in PBS) and pyrene actin (control), X - 20 μL of test buffer containing 20 μg

971 of occidiofungin and pyrene actin.

972 S6 Figure: Visualization of actin filaments: a) Untreated F-actin filaments stained with phalloidin

973 670 dye; Alkyne-OF treated F-actin filaments stained with azide derivatized AlexaFluor488 [(b)-

974 (40x); (c)- (100x)]

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FIGURE LEGENDS

Figure 1. Covalent structure of occidiofungin A-D and alkyne-OF.

Figure 2. *Candida albicans* morphology under hyphae inducing conditions. (A) The resulting morphology was scored as either 'yeast' or 'filamentous' at two hours and the resulting percent given. The data is presented as the average with the standard deviation for over 200 cells from each treatment condition (n=3). (B) The resulting cell morphology was scored as either 'yeast' (open circles) or 'filamentous' (closed triangles) after 0, 1, 2, 4, and 6 hours at 37°C. The data is presented as the average with the standard deviation for over 200 cells from each treatment condition (n=3). DMSO treated samples are represented by blue lines; Occidiofungin treated samples represented by red lines.

Figure 3. Effect of the native occidiofungin on endocytosis in fission yeast: DIC (top row) and fluorescence (bottom row) images of cells stained using FM-464 following treatment with sample blank (left column), 0.5x MIC of occidiofungin (middle column), and 1x MIC occidiofungin (last column). FM-464 dye uptake by endocytosis decreases in cells exposed to occidiofungin a dose dependent fashion.

Figure 4. Determination of *in vivo* interaction of occidiofungin: A) Representative samples obtained following affinity purification of whole cell extracts run on 12% SDS PAGE gels and stained with Coomassie blue (top) and silver staining (bottom). The Coomassie stained gel was run only until the proteins entered the separating phase whereas the silver stained gel was allowed to run completely. The bands in the Coomassie stained gel (demarcated by the arrows) were removed and used for LC-MS/MS analysis to determine the proteins. Broad range (10-250kDa) ladder was used on both gels; B) Cellular distribution of the proteins obtained in the pull-down assay following LC-MS/MS analysis.

Figure 5. *In vitro* interaction of occidiofungin with F- and G-actin: a) Affinity pulldown of actin using alkyne-OF: Lane 1- Ladder, Lane 2-100 ng pure F-actin, Lane 3-100 ng pure G-actin, Lane 4-Empty, Lane 5-F-actin treated with alkyne-OF, Lane 6-F-actin treated with native occidiofungin, Lane 7-F-actin treated with DMSO, Lane 8-G-actin treated with alkyne-OF, Lane 9-G-actin treated with native occidiofungin, Lane 10-G-actin treated with DMSO; b) Fluorescence microscopy analysis of the effect of occidiofungin treatment on actin filaments visualized using fluorescently labeled phalloidin: A: actin filaments treated with solvent blank (DMSO), B: Actin:native occidiofungin (24 µg actin:4 µg native occidiofungin), C: Actin:native occidiofungin (24 µg actin:8 µg native occidiofungin). Scale bar represents 5µm.

Fig 6. Isothermal titration calorimetry is used to measure occidiofungin (OF) binding to actin. A) Thermogram showing the heat exchange from equal injections of a solution containing OF into the ITC chamber containing actin. B) A representative binding isotherm of the integrated heat change from each injection shown in A is fit to a single-ligand binding model to yield an OF-actin dissociation constant. The mean and standard deviation from three independent experiments are $K_D = 1.0 \pm 0.8 \ \mu M$.

Fig 7. Co-sedimentation assay to demonstrate binding of occidiofungin to actin. A) Binding curve of phalloidin to actin (Kd = 8 nM and the stoichiometry (ligand : protein) is 0.6:1.0) and B) Binding curve of occidiofungin to actin (Kd = 1μM and the stoichiometry (ligand : protein) is 24:1). The graph is plotted between amount of free occidiofungin obtained in the supernatant of the co-sedimentation assay and the amount of bound occidiofungin obtained from the actin pellet. The data was fit to a standard Langmuir binding isotherm of the form: [X]bound = [X]*S/(Kd + [X]), where S is the maximal X bound, Kd is the dissociation constant and X is the concentration of free ligand.

Figure 8. Competition assay of native occidiofungin and alkyne-OF. Time course analysis (A-C) of alkyne-OF distribution and the distribution of alkyne-OF with the competition of native occidiofungin (D-F) in a) *Schizosaccharomyces pombe* and b) *Saccharomyces cerevisiae*. Arrows indicate specific localization patterns of alkyne-OF observed in each cell at 10, 30, and 60 minutes. When pretreated with native occidiofungin, alkyne-OF does not bind or is restricted to cellular envelope in *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*, respectively.

Figure 9. Effects of occidiofungin exposure on the integrity of actin cables in *S. cerevisiae* cells. A montage of cells processed for actin visualization using phalloidin-TRITC from: a) a culture exposed to solvent blank control (DMSO) where actin patches and cables are easily identifiable; and b) an occidiofungin treated culture (0.5X MIC; for 30 minutes) showing loss of actin cables and the accumulation of actin aggregates. Scale bars represent 2μm. The arrows are used to demarcate the presence of actin cables.

Figure 10. Efficacy of occidiofungin in treating murine vulvovaginal candidiasis. The graph demonstrates CFUS per ml of *Candida albicans* in the control group of mice compared to the groups treated intravaginally with different concentrations of occidiofungin in 0.3% noble agar. Error bars represent standard deviation.

Occidiofungin A: R_1 = H, R_2 = H, R_3 = H
Occidiofungin B: R_1 = OH, R_2 = H, R_3 = H
Occidiofungin C: R_1 = H, R_2 = Cl, R_3 = H
Occidiofungin D: R_1 = OH, R_2 = Cl, R_3 = H
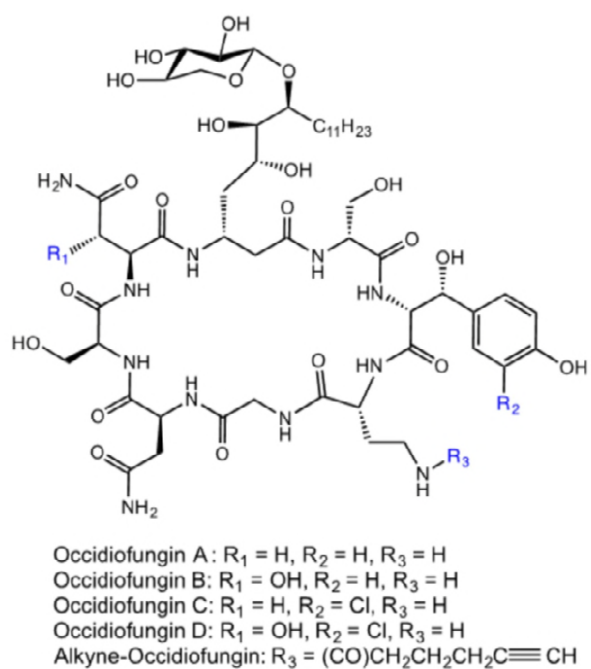Alkyne-Occidiofungin: R_3 = (CO)CH$_2$CH$_2$CH$_2$C≡CH

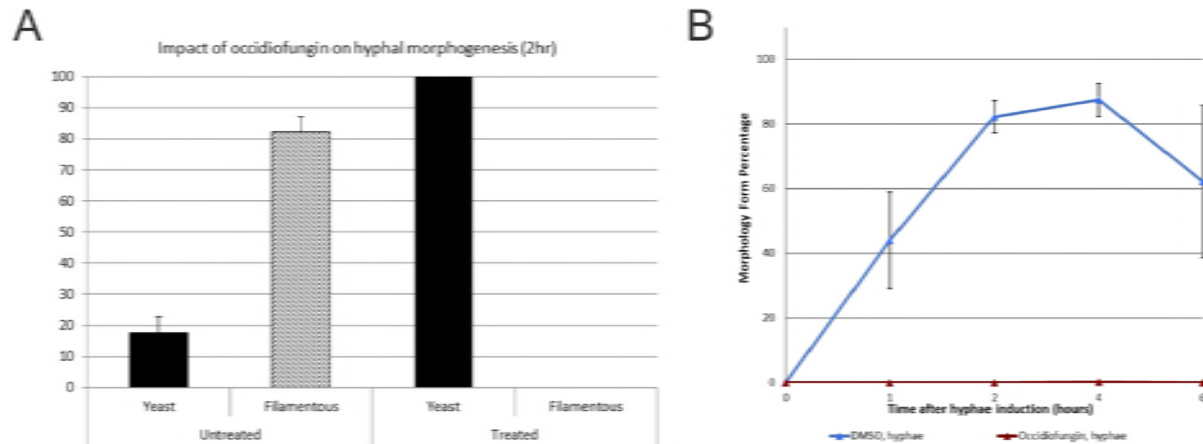Figure 1. Covalent structure of occidiofungin A-D and alkyne-OF.

Figure 2. *Candida albicans* morphology under hyphae inducing conditions. (A) The resulting morphology was scored as either 'yeast' or 'filamentous' at two hours and the resulting percent given. The data is presented as the average with the standard deviation for over 200 cells from each treatment condition (n=3). (B) The resulting cell morphology was scored as either 'yeast' (open circles) or 'filamentous' (closed triangles) after 0, 1, 2, 4, and 6 hours at 37°C. The data is presented as the average with the standard deviation for over 200 cells from each treatment condition (n=3). DMSO treated samples are represented by blue lines; Occidiofungin treated samples represented by red lines.

Figure 3. Effect of the native occidiofungin on endocytosis in fission yeast: DIC (top row) and fluorescence (bottom row) images of cells stained using FM-464 following treatment with sample blank (left column), 0.5x MIC of occidiofungin (middle column), and 1x MIC occidiofungin (last column). FM-464 dye uptake by endocytosis decreases in cells exposed to occidiofungin a dose dependent fashion.

Figure 4. Determination of *in vivo* interaction of occidiofungin: A) Representative samples obtained following affinity purification of whole cell extracts run on 12% SDS PAGE gels and stained with Coomassie blue (top) and silver staining (bottom). The Coomassie stained gel was run only until the proteins entered the separating phase whereas the silver stained gel was allowed to run completely. The bands in the Coomassie stained gel (demarcated by the arrows) were removed and used for LC-MS/MS analysis to determine the proteins. Broad range (10-250kDa) ladder was used on both gels; B) Cellular distribution of the proteins obtained in the pull-down assay following LC-MS/MS analysis.

Figure 5. *In vitro* interaction of occidiofungin with F- and G-actin: a) Affinity pulldown of actin using alkyne-OF: Lane 1- Ladder, Lane 2-100 ng pure F-actin, Lane 3-100 ng pure G-actin, Lane 4-Empty, Lane 5-F-actin treated with alkyne-OF, Lane 6-F-actin treated with native occidiofungin, Lane 7-F-actin treated with DMSO, Lane 8-G-actin treated with alkyne-OF, Lane 9-G-actin treated with native occidiofungin, Lane 10-G-actin treated with DMSO; b) Fluorescence microscopy analysis of the effect of occidiofungin treatment on actin filaments visualized using fluorescently labeled phalloidin: A: actin filaments treated with solvent blank (DMSO), B: Actin:native occidiofungin (24 µg actin:4 µg native occidiofungin), C: Actin:native occidiofungin (24 µg actin:8 µg native occidiofungin). Scale bar represents 5µm.

Fig 6. Isothermal titration calorimetry is used to measure occidiofungin (OF) binding to actin. A) Thermogram showing the heat exchange from equal injections of a solution containing OF into the ITC chamber containing actin. B) A representative binding isotherm of the integrated heat change from each injection shown in A is fit to a single-ligand binding model to yield an OF-actin dissociation constant. The mean and standard deviation from three independent experiments are $K_D = 1.0 \pm 0.8$ μM.
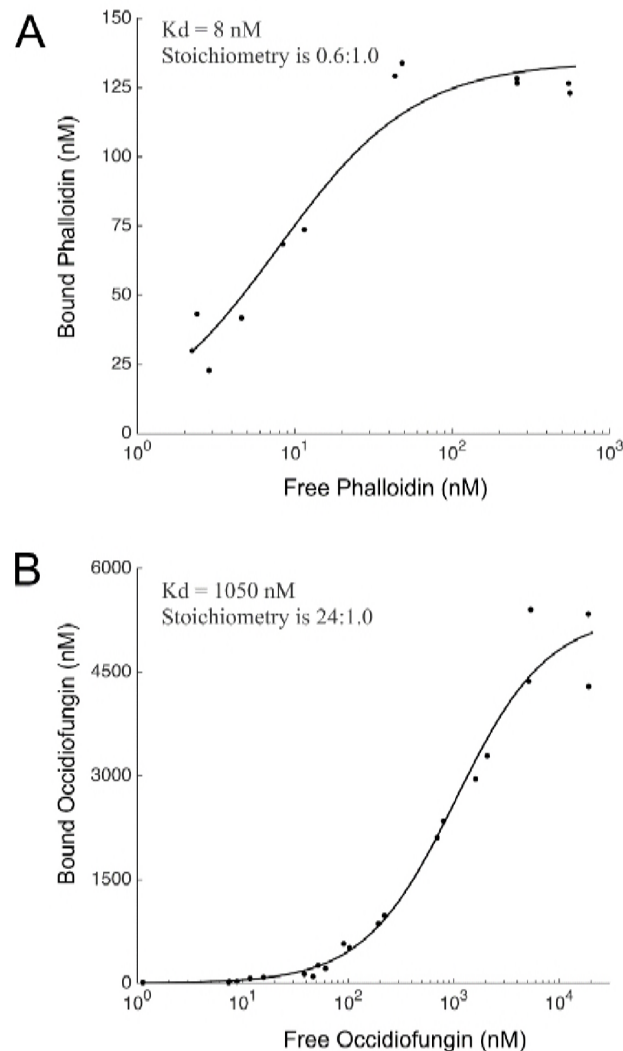
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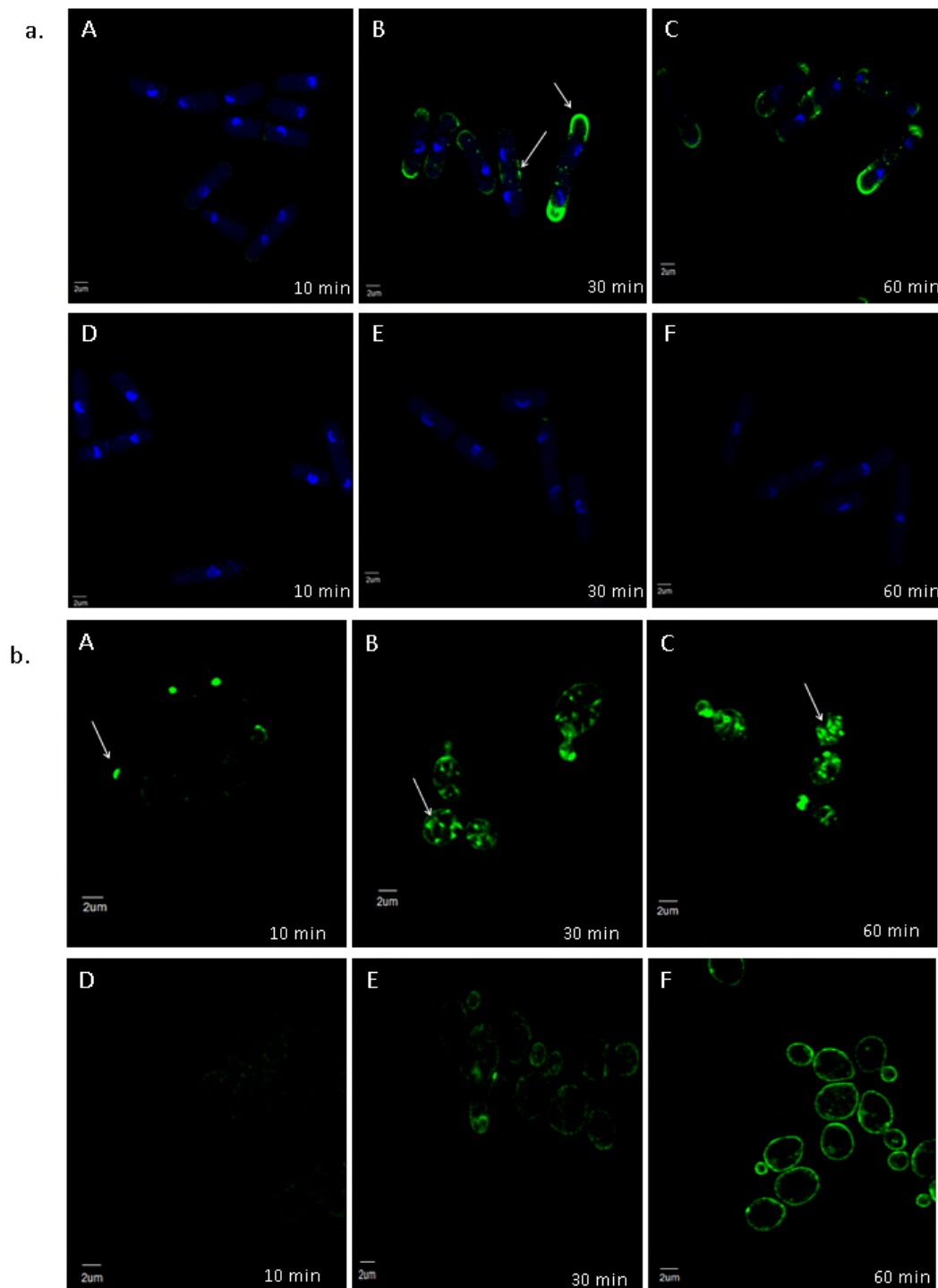
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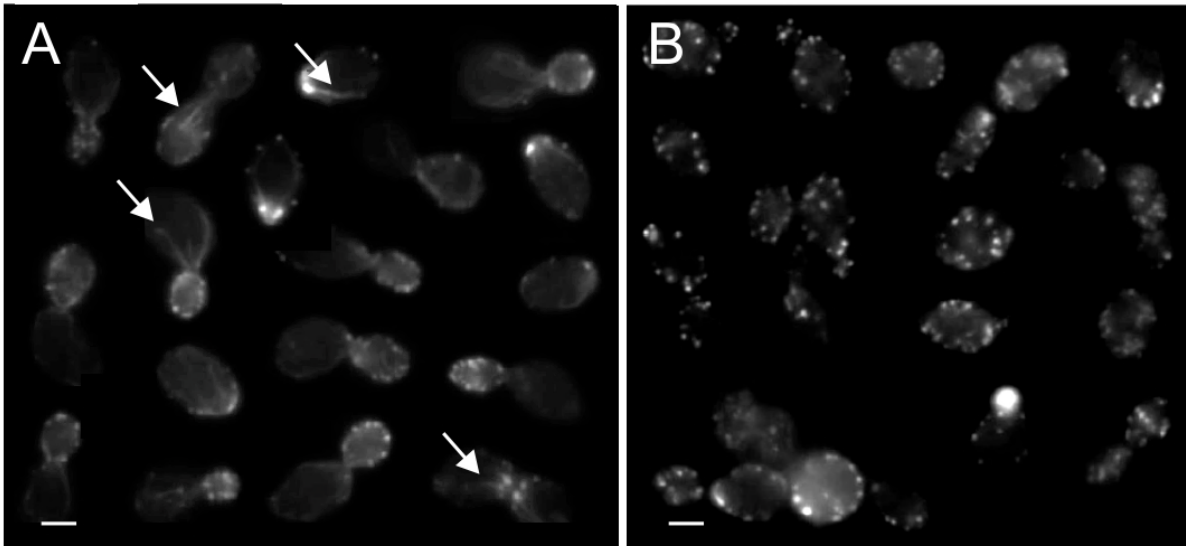
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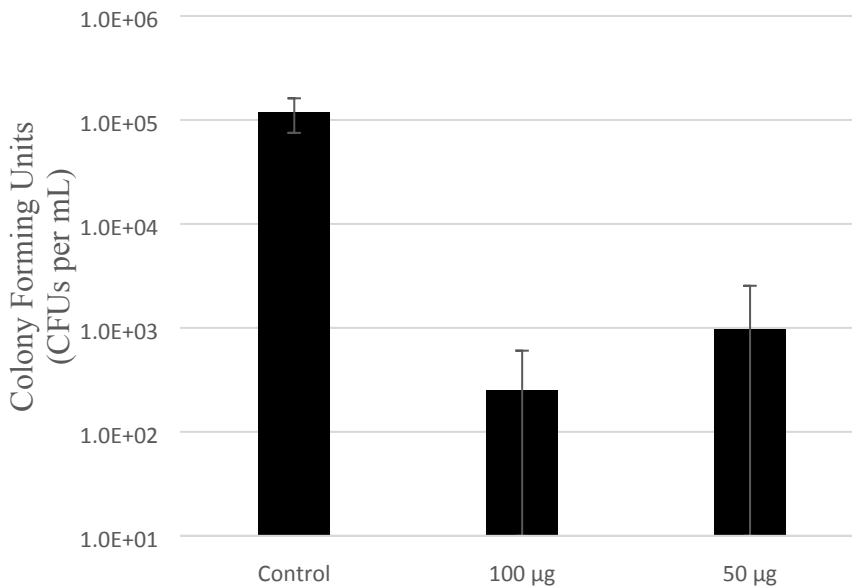
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