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# Identification of antifungal natural products via *Saccharomyces cerevisiae* bioassay: insights into macrotetrolide drug spectrum, potency and mode of action

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Since current antifungal drugs have not kept pace with the escalating medical demands of fungal infections, new, effective medications are required. However, antifungal drug discovery is hindered by the evolutionary similarity of mammalian and fungal cells, which results in fungal drug targets having human homologs and drug non-selectivity. The group III hybrid histidine kinases (HHKs) are an attractive drug target since they are conserved in fungi and absent in mammals. We used a *Saccharomyces cerevisiae* reporter strain that conditionally expresses HHK to establish a high-throughput bioassay to screen microbial extracts natural products for antifungals. We identified macrotetrolides, a group of related ionophores thought to exhibit restricted antifungal activity. In addition to confirming the use of this bioassay for the discovery of antifungal natural products, we demonstrated broader, more potent fungistatic activity of the macrotetrolides against multiple *Candida* spp., *Cryptococcus* spp., and *Candida albicans* in biofilms. Macrotetrolides were also active in an animal model of *C. albicans* biofilm, but were found to have inconsistent activity against fluconazole-resistant *C. albicans*, with most isolates resistant to this natural product. The macrotetrolides do not directly target HHKs, but their selective activity against *S. cerevisiae* grown in galactose (regardless of Drk1 expression) revealed potential new insight into the role of ion transport in the mode of action of these promising antifungal compounds. Thus, this simple, high-throughput bioassay permitted us to screen microbial extracts, identify natural products as antifungal drugs, and expand our understanding of the activity of macrotetrolides.

**Keywords** Fungi, reporter, natural products, drug discovery, macrotetrolide

## Introduction

The need for novel antifungal drugs has been escalating over the past 20 years. It is conservatively estimated that systemic fungal infections have increased over 200% between 1979 and 2000 [1]. The major factors contributing to this trend include the growing number of individuals with compromised immune systems, the complexity of medical procedures that render patients vulnerable, and the advancing age of our population [1]. Current antifungal drugs are insufficient due to the limited selection of medications, their adverse side-effects, and the emergence of resistance to them by fungal pathogens. A recent study reported that 17% of clinical isolates of *Candida albicans* had increased resistance to fluconazole, a current frontline drug [2]. Therefore, novel drugs are needed to meet the increasing demand for fungal infections.

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Natural products remain a great source of potential antifungal drugs as evidenced by the fact that both polyenes and echinocandins were derived from natural products of microbial origin [3,4]. However, a major drawback in the discovery of such drugs is the difficulty in identifying and purifying a compound from a complex extract of a natural product. In our ongoing effort to search for new and biologically active natural products produced by actinomycetes in unexplored and underexplored ecological niches [5–9], we assessed in this investigation the feasibility of a high-throughput bioassay to screen microbial extracts for antifungal natural products. We took advantage of a *Saccharomyces cerevisiae* yeast reporter strain that heterologously expresses a group IIII hybrid histidine kinase (HHK) to screen microbial extracts for antifungal activity. Group III HHKs are sensor kinases that regulate sporulation, virulence factors, and morphogenesis in numerous human fungal pathogens including *Aspergillus fumigatus*, *C. albicans*, *Cryptococcus neoformans*, and *Blastomyces dermatitidis* [10–13]. Conservation of HHKs throughout the fungal kingdom, together with their absence in mammals, makes them an attractive drug target.

Using this high-throughput reporter-cell bioassay, we successfully identified antifungal activities in products from a crude microbial extract. Dereplication and structural elucidation revealed that the antifungal activities resulted from the macrotetrolides, i.e., nonactin, monactin, dinactin, and trinactin, a family of ionphores previously thought to have only restricted antifungal action [14]. In addition to demonstrating the application of this bioassay, we uncovered that the macrotetrolides had broader, more potent activity against human fungal pathogens than previously appreciated. They were fungistatic against *C. albicans*, active against *C. neoformans and C. gatti* and fluconazole-resistant strains of *C. krusei*, as well as being effective *in vitro* and *in vivo* with respect to *Candida albicans* biofilms, a major source of life-threatening bloodstream infections. However, the majority of fluconazole-resistant *C. albicans* strains were resistant to the macrotretrolides. While they do not directly target HHKs, macrotetrolides selective activity against parental *S. cerevisiae* grown in galactose implies that potassium ion transport may have a pivotal role in the mode of action of this promising class of antifungal natural products.

**Table 1** Fungal strains used in this study.

| Organism | Notes | Reference/source |
|---|---|---|
| *Saccharomyces cerevisiae* | | |
| W303-1A | Parental; ATCC 208352 | ATCC |
| Hik1 Expressing | Hik1 under galactose promoter | Motoyama et al. 2005 [18] |
| Drk1 Expressing | Drk1 under galactose promoter | This study |
| *Candida albicans* | | |
| 412 | Clinical isolate | ATCC |
| 1002 | Clinical isolate | Andes et al. 2006 [37] |
| 2–76 | Clinical isolate | Andes et al. 2006 [37] |
| SC5314 | Clinical isolate | ATCC |
| C48 | Fluconazole-resistant clinical isolate | Sanglard et al. 1995 [38] |
| 2823 | Fluconazole-resistant clinical isolate | Andes et al. 2006 [37] |
| FH5 | Fluconazole-resistant clinical isolate | Andes et al. 2006 [37] |
| 2307 | Fluconazole-resistant clinical isolate | Andes et al. 2006 [37] |
| 12–99 | Fluconazole-resistant clinical isolate | Andes et al. 2006 [37] |
| 2168 | Fluconazole-resistant clinical isolate | This study |
| *Candida glabrata* | | |
| 1906 | Clinical isolate | This study |
| 1907 | Clinical isolate | This study |
| 1–215-8377 | Clinical isolate | This study |
| 1–218-8640 | Clinical isolate | This study |
| 3–218-8073 | Clinical isolate | This study |
| *Candida krusei* | | |
| 1685 | Clinical isolate | This study |
| 3459 | Clinical isolate | This study |
| 3–219-8544 | Clinical isolate | This study |
| *Candida lusitaniae* | | |
| 556 | Clinical isolate | This study |
| 3595 | Clinical isolate | This study |
| *Cryptococcus neoformans* var. *grubii* | | |
| H99 | Serotype A | ATCC |
| *Cryptococcus gattii* | | |
| 1266 | Clinical isolate | Personal communication* |
| 1269 | Clinical isolate | Personal communication* |
| *Aspergillus fumigatus* | | |
| PI 17 | Clinical isolate | This study |
| PI 18 | Clinical isolate | This study |
| PI 19 | Clinical isolate | This study |

*Strain kindly provided by Dee Carter from the University of Sydney, Australia.

## Materials and methods

### *Fungal strain and growth conditions*

The collection of fungal isolates used in this study is listed in Table 1, the majority of which represent human patient isolates. The *S. cerevisiae* reporter strain heterologously expresses Hik1, a group III HHK from *Magnaporthe grisea*, under the regulation of the *GAL1* galactose promoter (it was kindly provided by Takayuki Motoyama from RIKEN Wako, Japan) and was grown at 30°C. Yeast peptone dextrose (YPD) was used as a complete medium, and yeast synthetic complete (SC) served as a minimal medium [15]. *Candida* spp. cultures were maintained on YPD at 30°C,

while *Cryptococcus* spp. and *Aspergillus* spp. isolates were grown on YPD at 37°C.

Unless otherwise noted, supplies and reagents were purchased from Sigma-Aldrich or Fisher Scientific. Commercial antifungals were obtained from the pharmacy of the University of Wisconsin Hospital and Clinics, Madison, WI, USA.

### *Heterologous expression of Drk1 in* S. cerevisiae

cDNA was generated from *B. dermatitidis* RNA employing the iScript cDNA synthesis kit protocol (Bio-Rad) as discussed previously [16]. *B. dermatitidis* group III HHK *DRK1* (dimorphism regulating kinase) was PCR amplified from cDNA template using the following primers (5-gg ggacaagtttgtacaaaaaagcaggctaaaaatgactcggggtgatga aac-3 and 5-ggggaccactttgtacaagaaagctgggtctaatctttagtcc acgaac-3). *DRK1* was inserted into the pDONR vector (Invitrogen) following the Gateway cloning technology protocol (Invitrogen). PCR-induced *DRK1* mutations were repaired using the QuickChange II-E Site-Directed Mutagenesis Kit (Stratagene), and the corrections were verified by sequencing. *DRK1* was then inserted into the pYES-DEST52 vector (Invitrogen) under the control of the galactose promoter following the Gateway cloning technology protocol (Invitrogen). *S. cerevisiae* was transformed with the pYES-DEST52-*DRK1* vector using uracil selection as described in a prior publication [17]. Sensitivity of this *S. cerevisiae* reporter to fludioxonil following conditional expression of *DRK1* was verified as described [18].

### *Generation of microbial crude extracts*

A collection of 190 actinomycete strains from selected ecological niches in various parts of China were grown in one to seven media to afford a total of 897 microbial extracts. Each strain was first grown in 18 × 150 mm glass tubes to prepare the seed culture (5 ml), which were then used to inoculate two 50 ml cultures in 250-ml baffled Erlenmyer flasks, which were incubated at 250 rpm at 28°C for 7 days. Following fermentation, 2.5 g of XAD-16 resin was added to each flask, and the flasks were incubated for an additional 2 h. The contents of each flask was transferred to a 50-ml tube and centrifuged to pellet the resin and cells in a bench-top centrifuge. The resulting pellets from the duplicate fermentation flasks were combined and washed twice with 20 ml distilled water. The washed resin and cell pellets were frozen on dry ice and lyophilized overnight. The dried pellet was extracted twice with 20 ml methanol, the solvent was removed from the combined methanol extracts with a Genevac centrifuge evaporator, and the remaining material was suspended in 1 ml DMSO to generate a crude microbial natural product extract.

### *High-throughput screen of microbial extracts*

The microbial extracts were screened in two steps. First, the *S. cerevisiae* reporter strain, with Hik1 under the control of a galactose-inducible promoter, was added to 96-well plates with SC medium lacking uracil and containing galactose at 0.1 $OD_{600nm}$/well. The microbial extracts were added to wells, with the medium and fludioxonil serving as negative and positive controls, respectively. The plate was incubated at 30°C overnight and growth was quantified by measuring the optical density at $OD_{600nm}$ with a plate reader. Extracts that caused a growth reduction $\geq 50\%$ relative to the growth control were carried forward to the next screening step. To confirm activity against group III HHKs, active extracts were tested against parental and Hik1-expressing *S. cerevisiae* strains. Extracts that resulted in $\geq 50\%$ growth reduction of the Hik1-expressing strain, but $\leq 10\%$ reduction of the parental strain were subjected to dereplication to isolate the active natural products.

### *Dereplication, purification, and structure elucidation of macrotetrolides as antifungal natural products from* Streptomyces *sp. YIM56295*

*Streptomyces* sp. YIM56295 was isolated in Yunnan province in the southwest of China from the leaf of *Diphylleia sinensis*, a traditional Chinese medicinal plant widely used to reduce inflammation. The strain was preserved as a spore solution at −80°C. To dereplicate the antifungal natural products from *Streptomyces* sp. YIM56295, the strain was cultured in D medium (yeast extract 0.2%, malt extract 0.5%, dextrose 0.2%, pH 7.0) by a two-stage fermentation process. Inoculum was prepared in a 250-ml baffled Erlenmeyer flask, containing 50 ml of D-medium, inoculated with 10 µl of the *Streptomyces* sp. YIM56295 spore solution, and incubated at 250 rpm for 2 days as 28°C. Eighteen 2-l baffled Erlenmeyer flasks, each containing 400 ml of D-medium, were then inoculated with 20 ml from separate 50 ml seed cultures and grown for 7 days under identical conditions.

The contents of each of these production cultures was centrifuged at 5,000 rpm at 4°C for 30 min to remove mycelia, and the remaining broth was incubated with 3% Amberlite XAD16 resin for 4 h at room temperature with agitation. The resin was harvested by centrifugation, eluted three times with methanol, with the combined elution concentrated under reduced pressure to generate the crude extract.

The crude extract (3.8 g) was subjected to silica gel chromatography, eluted with a stepwise chloroform:methanol gradient using 1 l of each of the following ratios, 100:0, 50:1, 20:1, 10:1, 5:1 and 0:100, to generate six fractions, i.e., A to F. Only fraction E had activity when it was

screened for group III HHK-dependent activity against *S. cerevisiae*. Fraction E (119 mg) was then purified by semi-preparative HPLC on an Alltima-C18 column (5 μm; 9.4 mm × 25 cm), eluted with a 30-min solvent gradient (3 ml/min) from 20% methanol in $H_2O$-0.1% formic acid to 90% methanol in $H_2O$-0.1% formic acid to generate compounds 1 (2.7 mg), 2 (3.1 mg), 3 (8.0 mg), and 4 (7.0 mg), all of which were active in the *S. cerevisiae* reporter bioassay.

High-resolution electrospray ionization Fourier transform mass spectrometry (ESI-FTMS) and $^{13}C$ and $^{1}H$ NMR spectroscopy structural analysis of these purified compounds was performed as described in an earlier publication [19].

### *Determination of minimal inhibitory concentration (MIC)*

The MIC of the macrotetrolides against *Candida* spp. and *Cryptococcus* spp. was determined in accord with the Clinical Laboratory and Standards Institute (CLSI) protocol M27-A3. Briefly, overnight liquid cultures of yeast were enumerated using a hemocytometer, suspended in RPMI-1640 buffered to pH 7.0 with MOPS (RPMI-1640/MOPS) to a density of $3 \times 10^3$ yeast/ml, and 100 μl of cells was added to each well of 96-well plates containing media and titrated drug in triplicate. The plates were incubated overnight at 37°C, and the MIC was defined as the lowest concentration that prevented visible growth. The MIC of the macrotetrolides against *Aspergillus* was determined following CLSI protocol M38-A2, which is identical to M27-A3 except that the inoculum was at $2 \times 10^4$ spores/ml. The reported MICs are representative of at least two independent experiments.

### *Determination of fungicidal vs. fungistatic action*

The cidal activity of the macrotetrolides was determined by quantifying colony forming units (CFU)/ml. The cells from MIC wells were suspended and 100 μl were inoculated onto YPD plates. Cells from the yeast only well served as a 'no compound' control, with 100 μl of each well dilution spread onto a YPD plate. Plates were incubated overnight at 37°C, and the number of colonies enumerated. Fungicidal activity was defined as a >99.99% reduction in the numbers of colonies in comparison to the results obtained with the 'no compound' control.

### *Candida albicans* in vitro *biofilm assay*

Macrotetrolide activity against *in vitro Candida* biofilm was assessed as described earlier [20]. Briefly, the numbers of cells in overnight cultures of *C. albicans* (strain SC5314) were enumerated using a hemocytometer. The yeasts were suspended in RPMI-1640/MOPS at $1 \times 10^6$ yeast/ml and 100 μl was added to each well of a flat bottom 96-well plate and incubated at 37°C for 24 h. The plate was gently washed twice with PBS. The macrotetrolides were added to the plate in two-fold dilutions ranging from 0.05–25 μg/ml. Media and solvent served as the negative controls, and wells with fluconazole or amphotericin B as positive controls. Samples were tested in triplicate. After overnight incubation at 37°C, the plate was washed twice with PBS. Biofilm viability was quantified with the XTT (2,3-bis [2-methoxy-4-nitro-5-sulfophenyl]-5-[phenylamino] carbonyl]-2H-tetrazolium hydroxide) assay (Sigma) as described in a previous publication [21]. Growth reduction was determined relative to the media control.

### *Determination of* C. albicans hyphae *sensitivity to antifungals*

*C. albicans* hyphae were generated as described by van der Graaf and co-workers [22]. Briefly, *C. albicans* (strain SC5314) was grown in RPMI-1640 media adjusted to pH 6.4 with hydrochloric acid for 24 h at 37°C. After incubation, microscopic examination revealed that >95% of the cells were hyphae/pseudohyphae. Compound MIC was determined following the same protocol as with *C. albicans* yeast, except that flat-bottomed 96-well plates were used.

### In vivo *rat-catheter model of* C. albicans biofilm

The *in vivo* efficacy of dinactin was assayed using a rat catheter model of *C. albicans* biofilm as has been discussed [23]. Briefly, a heparinized (100 U/ml) polyethylene catheter was surgically implanted into the external jugular vein of a ~400 g specific-pathogen-free Sprague-Dawley rat (Harlan Laboratories, Madison, WI, USA). After a 24-h recovery period, the catheter was inoculated with 500 μl (strain K1) of a $10^6$ yeast/ml suspension of *C. albicans*. After 6-h incubation, the catheter was flushed to remove unattached *C. albicans*, and incubated for an additional 24 h to enable the formation of a biofilm. Drug delivered into the catheter was then allowed to dwell for 24 h. Control catheters were treated with PBS. After sacrificing the animals, catheters were aseptically removed, placed in sterile saline, sonicated for 10 min, and vortexed. The burden of *C. albicans* was quantified by measuring CFU on Sabouraud dextrose agar (SDA) after incubation at 30°C overnight.

## Results

### *A high-throughput screen of microbial extracts identifies natural products with group III HHK-dependent antifungal activity*

Microbial extracts were screened in a high-throughput manner against the Hik1-expressing *S. cerevisiae* strain

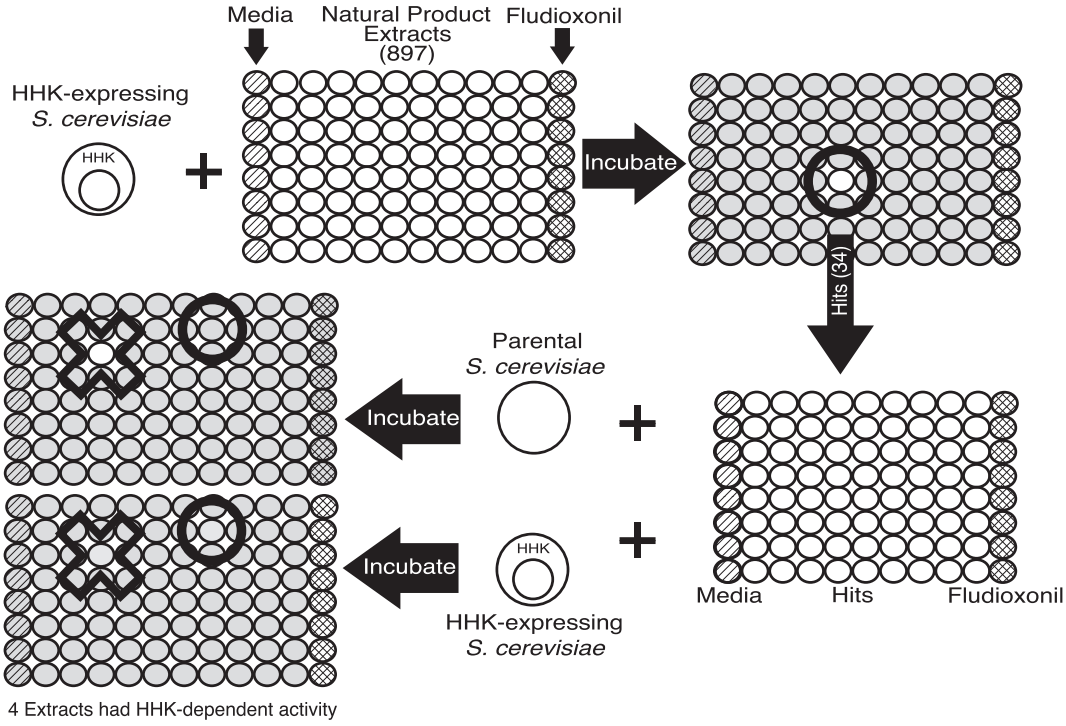



**Fig. 1** HHK-*Saccharomyces cerevisiae* natural product extract screen. The HHK-*S. cerevisiae* strain was seeded at 0.1OD600 nm in 96-well plates containing natural product extracts. Media and fludioxonil served as positive and negative controls respectively. After overnight incubation at 30°C, extracts that inhibited growth >50% (based on OD600 nm) were considered hits (circle). These hits were assayed against the HHK-expressing and parental *S. cerevisiae* strains to determine if they had HHK-dependent activity in a secondary screen. After overnight incubation at 30°C, extracts that inhibited the growth of both strains >50% were discarded (cross). Extracts that inhibited the growth of the HHK-expressing *S. cerevisiae* >50% but did not prevent the growth of the parental strain (circle) were considered hits and subjected to fractionation.

(Fig. 1). The compound fludioxonil served as a positive control since its mode of action is known to require the expression of group III HHKs [18]. In the primary screen, fludioxonil and 34 (3.8%) of the 897 natural product extracts assayed caused >50% growth reduction of the Hik1-expressing yeast as compared to treatment with the solvent control (data not shown). To test the group III HHK-dependence of this growth inhibition, a secondary screen was performed to determine whether the parental *S. cerevisiae* was inhibited by the extracts. In this secondary screen, fludioxonil and four of the 34 extract hits inhibited growth of the Hik1-expressing strain by >50%, but did not inhibit growth of parental *S. cerevisiae* growth by >10% (data not shown), suggesting that the growth inhibition was group III HHK-dependent.

We de-replicated the natural products from one of the four active extracts responsible for HIK1-dependent activity, which in turn led to the isolation and purification of four compounds. High-resolution electrospray ionization Fourier transform mass spectrometric (ESI-FTMS) analysis revealed the molecular formulas of the four compounds (Table 2). Comparison of these formulas with the Sci-finder database disclosed the macrotetrolides nonactin, monactin, dinactin, and trinactin as likely structural hits. The ESI-FTMS results were confirmed by comparing $^{1}H$ and $^{13}C$ NMR spectroscopic data of the purified compounds with literature values (Supplementary Fig. 1 to be found online at http://www.informahealthcare.com/doi/abs/10.3109/13693786.2012.710917) [24–26]. Compounds 1–4 were thus confirmed as the macrotetrolides nonactin, monactin, dinactin, and trinactin (Fig. 2), which have been isolated previously from various actinomycetes (24–26) and are known ionophores [27].

### *Monactin, dinactin, and trinactin exert potent activity against multiple fungal pathogens*

To determine the antifungal spectrum and potency of the macrotetrolides, we measured their MICs against clinical isolates of pathogenic fungi (Table 3). MIC values of 3.13, 1.56–6.25, and 1.56–3.13 μg/ml for monactin, dinactin and trinactin, respectively, were found in tests with *C. albicans* (Table 3). These effective concentrations were close to the MICs observed for the commercial antifungal drugs fluconazole and amphotericin B, which had MICs of 0.78–1.56 and 0.39–0.78 μg/ml, respectively (Table 3). Inoculating a portion of the MIC wells of a representative

**Table 2** Mass spectrometry analysis of the antifungal natural products isolated from *Streptomyces* sp. YIM56295.*

| Compound | Theoretical mass | Observed mass | Formula | Identity |
|---|---|---|---|---|
| 1 | 759.4295 | 759.4290 | $C_{40}H_{64}O_{12}Na$ | Nonactin |
| 2 | 773.4451 | 773.4462 | $C_{41}H_{66}O_{12}Na$ | Monactin |
| 3 | 787.4608 | 787.4583 | $C_{42}H_{68}O_{12}Na$ | Dinactin |
| 4 | 801.4764 | 801.4780 | $C_{43}H_{70}O_{12}Na$ | Trinactin |

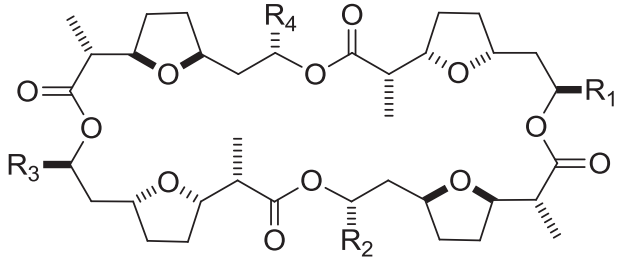
*See Supplementary Fig. 1 for $^{1}H$ and $^{13}C$ NMR spectra confirming the structures of these natural products.

macrotetrolide-sensitive isolate revealed that the macrotetrolides were fungistatic (although % killing was >99.7%; data not shown).

*Candida krusei* (fluconazole MIC >25 μg/ml), which is naturally resistant to fluconazole, was consistently more sensitive to monactin, dinactin, and trinactin, having corresponding MICs of 1.56–12.5, 1.56–12.5, and 1.56–>25 μg/ml (Table 3). Of six fluconazole-resistant isolates of *C. albicans*, one was sensitive to the macrotetrolides and had monactin, dinactin, and trinactin MICs of 3.13, 1.56, and 1.56 μg/ml, respectively (Table 3). The other five fluconazole-resistant isolates were also resistant to the macrotetrolides.

Monactin, dinactin, and trinactin had potent activity against both clinically important *Cryptococcus* species, which are the most common cause of fungal meningitis [28]. *Cryptococcus gatti* is emerging in non-immune compromised populations and is often resistant to fluconazole [29]. Both isolates of *C. gatti* used in these studies were sensitive, having MICs of 3.13, 1.56, and 1.56 μg/ml for monactin, dinactin, and trinactin respectively (Table 3). *Cryptococcus neoformans* var. *grubii* had the same MIC of 3.13 μg/mL for monactin, dinactin, and trinactin (Table 3).

Monactin, dinactin, and trinactin exhibited limited activity against *C. glabrata*, *C. lusitaniae*, and *A. fumigatus* at any of the concentrations tested, up to 25 μg/ml (Table 3).




Nonactin (1): $R_1 = R_2 = R_3 = R_4 = CH_3$
Monactin (2): $R_1 = CH_2CH_3$, $R_2 = R_3 = R_4 = CH_3$
Dinactin (3): $R_1 = R_3 = CH_2CH_3$, $R_2 = R_4 = CH_3$
Trinactin (4): $R_1 = R_2 = R_3 = CH_2CH_3$, $R_4 = CH$

**Fig. 2** Macrotetrolides isolated from the extract of *Streptomyces* sp. YIM56295.

### *Monactin, dinactin, and trinactin exert potent activity against a* Candida albicans *biofilm*

*C. albicans* is able to form biofilms on implanted devices such as central venous catheters, which provides it a critical niche for growth and pathogenesis. These biofilms are difficult to treat due to their resistance to fluconazole and represent a major source of deadly bloodstream infections. In light of the potent activity of the macrotetrolides against planktonic growth of *C. albicans*, we investigated their activity against *C. albicans* in biofilms in a widely used *in vitro* model. Remarkably, monactin, dinactin, and trinactin each had potent activity against *C. albicans* biofilms, showing >50% growth reduction at drug concentrations of less than 5 μg/ml (Fig. 3). In contrast, fluconazole at a 100-fold higher concentration failed to inhibit biofilm growth by more than 20% (Fig. 3).

*C. albicans* biofilm contains yeast, pseudohyphae, and hyphae [30]. The macrotetrolides had robust activity against yeast cells in our MIC studies of planktonic *C. albicans* (Table 3) and its hyphe and pseudohyphae, as evidenced in the biofilm studies, showed the same sensitivity as yeast cells to the macrotetrolides (data not shown).

### *Activity of macrotetrolides against* in vivo C. albicans *biofilm*

The robust *in vitro* activity of the macrotetrolides against *C. albicans* in biofilms warranted evaluating the compounds *in vivo* efficacy against a *C. albicans* biofilm. We studied the activity of dinactin in a rat catheter model of *C. albicans* biofilm infection and found that at 20 μg/ml it reduced the catheter fungal burden by 29% compared to control (from $3.8 \times 10^6$ to $2.7 \times 10^6$ CFU/ml). A higher dinactin dose of 100 μg/ml reduced the fungal burden of two treated animals by an average of 77% relative to control (from $2.7 \times 10^6$ to $6.46 \times 10^5$ and $5.3 \times 10^5$ CFU/ml, respectively), but intravenous administration of 100 μg/ml of dinactin was poorly tolerated by the animal (lethal after 24 h).

### *Mode of action of the macrotetrolides against HHK-expressing* S. cerevisiae

Once the macrotetrolides had been purified in quantity from the *Streptomyces* sp. YIM56295 culture, we sought to retest their group III HHK-dependent growth inhibition. To do this, the MIC of the macrotetrolides against the galactose-induced reporter and parental strains of *S. cerevisiae* were determined in a medium containing glucose or galactose (Table 4). For these experiments, the group IIII

**Table 3** Macrotetrolide spectrum of activity against the pathogenic fungi *Candida*, *Cryptococcus,* and *Aspergillus.**

| Organism | Nonactin | Monactin | Dinactin | Trinactin | Fluconazole | Amphotericin B |
|---|---|---|---|---|---|---|
| *Candida albicans* wild type (4) | >25¥ | 3.13¥ | 1.56–6.25 | 1.56–3.13 | 0.78–1.56 | 0.40–0.78 |
| *Candida albicans* fluconazole-resistant (6) | >25Σ | 3.13–>25Σ$^{\Omega}$ | 1.56 –>25$^{\Omega}$ | 1.56–>25$^{\Omega}$ | >25 | 0.40–0.78 |
| *Candida glabrata* (5) | >25 | >25 | >25 | >25 | 3.13–>25 | <0.05–0.20 |
| *Candida krusei* (3) | >25 | 1.56–12.5 | 1.56–12.5 | 1.56–>25$^{\dagger}$ | >25 | 0.40 |
| *Candida lusitaniae* (2) | >25 | >25 | >25 | >25 | 0.78–1.56 | 0.10 |
| *Cryptococcus neoformans* var. *grubii* (1) | >25 | 3.13 | 3.13 | 3.13 | 3.13 | 0.05 |
| *Cryptococcus gattii* (2) | >25 | 3.13 | 1.56 | 1.56 | 0.78 | 0.40–0.78 |
| *Aspergillus fumigatus* (3) | >25 | >25 | >25 | >25 | NT$^{+}$ | 0.40–0.78 |

*Microbroth dilution quantification of compound MIC (μg/ml) against yeast and filamentous fungal pathogens. The number of fungal strain isolates tested is in parentheses. The values are representative of at least two independent experiments.
¥This compound was assayed against three of the four isolates.
ΣThis compound was assayed against two of the six isolates.
$^{\Omega}$Five of the six isolates tested had MICs > 25 μg/ml.
$^{\dagger}$The *C. krusei* isolate MICs were 1.56, 3.13, and > 25 μg/ml.
$^{+}$NT = not tested.



HHK-dependent compound fludioxonil served as a positive control (Table 4). For example, the parental strain (lacking a group III HHK) was resistant to fludioxonil (MIC > 50 μg/ml) in glucose and galactose media (Table 4). In contrast, the group III HHK-expressing strain was resistant to fludioxonil when grown in glucose (MIC > 50 μg/ml), and sensitive to the compound (MIC of 1.56 μg/ml) in galactose (Table 4).

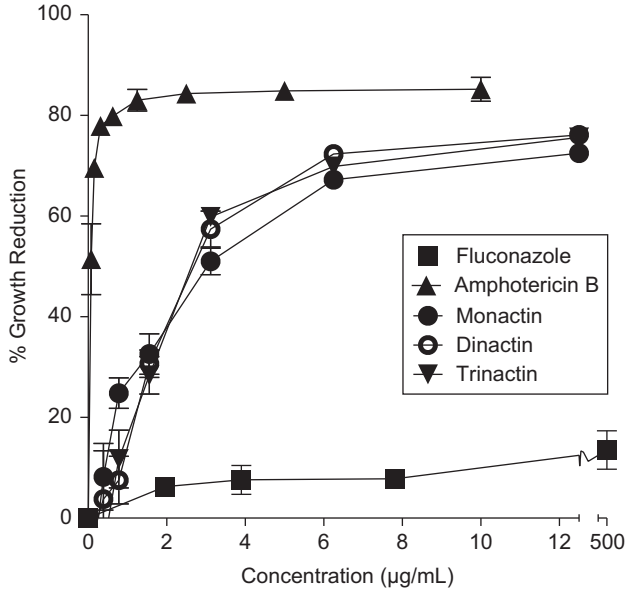



**Fig. 3** Monactin, dinactin, and trinactin had robust activity against *Candida albicans* biofilm. *In vitro C. albicans* biofilm (24 hour) was incubated with titrations of monactin, dinactin, and trinactin (0.40–12.5 μg/ml), amphotericin B (0.08–10 μg/ml), and fluconazole (1.95–500 μg/ml) overnight at 37°C. Biofilm viability was measured using the XTT assay relative to media-exposed cells. These data are represented as the mean ± SD of three samples per treatment group, which are representative of three experiments.

As expected, both the parental strain and group III HHK reporter strain were resistant to the macrotetrolides in glucose medium (Table 4). Although the reporter strain became extremely sensitive to the macrotetrolides in galactose media with MICs of 25, 0.78, 0.39, and 0.39 μg/ml for nonactin, monactin, dinactin, and trinactin, respectively (Table 4), the parental strain also was unexpectedly sensitive to the macrotetrolides when grown in galactose. Both strains grew well in the presence of galactose without macrotetrolides (data not shown). Thus, the sensitivity of *S. cerevisiae* to the macrotetrolides was carbon-source dependent, arguing that macrotetrolides may not act directly upon group III HHKs. This dependency of activity upon carbon source was distinct for the macrotetrolide family of natural products, as we have not observed a similar phenomenon in screening and identifying other small molecules with HHK-dependent activity against the reporter strain (data not shown).

**Table 4** Macrotetrolide sensitivity is carbon source-dependent in *Saccharomyces cerevisiae* but not *Candida albicans.**

| Organism | Nonactin | Monactin | Dinactin | Trinactin |
|---|---|---|---|---|
| III HHK *S. cerevisiae* (glucose) | >50 | >50 | >50 | >50 |
| III HHK *S. cerevisiae* (galactose) | 25 | 0.78 | 0.39 | 0.39 |
| Wild type *S. cerevisiae* (glucose) | >50 | >50 | >50 | >50 |
| Wild type *S. cerevisiae* (galactose) | 25 | 0.78 | 0.39 | 0.39 |
| *C. albicans* (glucose) | NT$^{+}$ | NT | 2.5 | 20 |
| *C. albicans* (galactose) | NT | NT | 2.5 | 20 |

*Microbroth dilution quantification of compound MICs (μg/ml) with III HHK-expressing and wild type *S. cerevisiae* and *C.albicans* in glucose and galactose. The values are representative of at least two independent experiments. $^{+}$NT = not tested.

Distinct features of potassium flux in *S. cerevisiae* yeast, fostering greater uptake in the presence of glucose, may have combined with the ionophore like properties of the macrotetrolides to render *S. cerevisiae* sensitive to this class of compounds [31]. To see if the activity of macrotetrolides on human fungal pathogens is simply conditional and dependent on carbon source, we tested the activity of the macrotetrolides against *C. albicans* grown in glucose and galactose. Dinactin is active against *C. albicans* in both media. Thus, *C. albicans* sensitivity to the macrotetrolides is independent of carbon source (Table 4).

We also tested the drug sensitivity of the parental and Drk1-expressing *S. cerevisiae* strains in RPMI-1640/MOPS to ensure that this medium was not responsible for the difference in sensitivity between *C. albicans* and *S. cerevisiae*. Both *S. cerevisiae* strains were resistant (MIC $>25$ μg/ml) to fludioxonil, dinactin, and trinactin in this medium (data not shown).

## Discussion

Morbidity and mortality associated with fungal infections have increased in recent decades, but current drugs are unable to meet the growing demand for antifungal therapeutics. We exploited the group III HHK-expressing *S. cerevisiae* reporter strain to establish a simple high-throughput bioassay to quickly screen microbial extracts, made from actinomycetes isolated from unexplored and underexplored ecological niches, to identify novel antifungal natural products. Our assay successfully detected antifungal microbial extracts with potent, broad-spectrum activity. Dereplication of one of the active extracts, made from *Streptomyces* sp. YIM56295, resulted in the identification of the macrotetrolides (nonactin, monactin, dinactin, and trinactin) as responsible for the observed antifungal activity. Whereas the macrotetrolides may not act directly upon group III HHKs, we obtained novel insights about the antifungal properties of this class of compounds, including broader and more potent activity than previously appreciated, and possible new insight about their mode of action.

The macrotetrolides are a group of closely related compounds produced by various *Streptomyces* species (24–26). They are broadly active antimicrobials which affect bacteria, fungi, and mites [32]. Macrotetrolides were initially reported to have only limited activity against *C. albicans and C. neoformans*. We investigated by disk diffusion whether they might have broader and more potent activity against fungal pathogens than previously reported because the macrotetrolides showed such potent activity in our high-throughput bioassay [32].

We found here that monactin, dinactin, and trinactin had robust activity against clinical isolates of *C. albicans*, *Cryptococcus*, and fluconazole resistant *C. krusei* and *C. albicans*. The purity of the macrotetrolides used and the different fungal isolates in our studies are two possible explanations for the difference between our results and those reported by Ando *et al*. [32]. However, the most likely cause for the disparity is the method that they employed to assess antifungal activity. These authors [32] used the agar dilution method and noted that the macrotetrolides were insoluble in water, resulting in very small zones of growth inhibition. We utilized the microbroth dilution method to quantify the activities of the macrotetrolides and observed no solubility problems. We recognize that our studies represent a relatively small sampling of clinical isolates and that our findings need to be confirmed with a larger sample size of clinical isolates. Consistent with prior reports [14], nonactin showed no activity in our study.

*C. albicans* biofilms on implanted medical devices are a major source of life-threatening blood stream infections. Monactin, dinactin, and trinactin had robust *in vitro* activity against *C. albicans* in biofilms. Fluconazole, a frontline drug for the treatment of *C. albicans* infections, was relatively ineffective against the yeast in such biofilms, while amphotericin B was active against them. Dinactin was active in a rat catheter model of *C. albicans* infection but the animals did not tolerate intravenous administration at 100 μg/ml. However, the macrotetrolides are well tolerated by vertebrates via other routes of administration. The $LD_{50}$ for mice is $>300$ mg/kg after intraperitoneal delivery and $>1500$ mg/kg following oral administration [32]. Although our preliminary *in vivo* results demonstrated activity of dinactin against *C. albicans* biofilm, pharmacokinetic studies are needed to improve these attractive drug leads for treatment of *C. albicans* biofilms.

Our analysis of the antifungal action of the macrotetrolides revealed that they did not act directly on group III HHKs. We observed that *S. cerevisiae* grown on glucose enabled this yeast to resist the macrotetrolides, whereas growth on galactose significantly enhanced its sensitivity to them. Moreover, the parental and Drk1-expressing *S. cerevisiae* strains were equally sensitive to the macrotetrodlides, suggesting that the sensitivity of *S. cerevisiae* is dependent on the carbon source of the media, not Drk1 expression. Since the macrotetrolides are known to be ionophores capable of transporting potassium across lipid membranes [33], a possible explanation for these findings is the effect of carbon source on potassium transport in *S. cerevisiae*. Since glucose stimulates potassium uptake in *S. cerevisiae* [34], we hypothesize that the macrotetrolides promote death of yeast by depleting their intracellular potassium stores. The increased potassium uptake of *S. cerevisiae* grown on glucose may buffer the cells against potassium loss, whereas yeast grown on galactose would be unable to replenish their potassium stores.

The leakage of potassium is thought to be involved in the mechanism of action of amphotericin B in *C. albicans* [35,36]. However, the loss of intracellular potassium is not likely involved in macrotetrolides activity against *C. albicans* since the compounds exerted their activity against *C. albicans* independent of the carbon source.

Macrotetrolides were inactive against five out of six of the fluconazole-resistant *C. albicans* isolates tested. Only fluconazole-resistant isolate 2823 was sensitive to the macrotetrolides. This strain over-expresses Mdr1, a major facilitator superfamily protein, and Erg11, a cytochrome P-450 lanosterol 14-alpha-demethylase, the enzyme target of fluconazole [37]. The fact that over-expression of Erg11 does not engender resistance to the macrotetrolides indicates that it is unlikely that the compounds target ergosterol biosynthesis in the same manner as fluconazole.

The mechanism of resistance to fluconazole is known for three of the five macrotetrolide-resistant strains of *C. albicans*. *C. albicans* strain C48 over-expresses Erg11 and Cdr1, an ATP-Binding Cassette (ABC) transporter drug efflux pump [38]; isolate 12–99 over-expresses Cdr1, Mdr1 and Erg11; and strain FH5 over-expresses Cdr1 [37]. The common phenotype of these macrotetrolide-resistant *C. albicans* isolates is the over-expression of Cdr1. As a drug efflux pump, Cdr1 can shuttle a wide variety of compounds across the plasma membrane [39]. In contrast, Mdr1 is thought to be capable of causing the efflux of only fluconazole [40]. Therefore, the decreased activity of the macrotetrolides against *C. albicans* isolates that over-express Cdr1 is likely due to the drug efflux pumps preventing accumulation of macrotetrolide within the cell.

In summary, we developed a simple, high-throughput, yeast bioassay and demonstrated its utility in discovering natural products as novel antifungal drug leads, as exemplified by the isolation of the macrotetrolides from *Streptomyces* sp. YIM56295. Our results should be interpreted with caution due to the limited number of strains available for testing in our study. This point notwithstanding, the macrotetrolides have potent activity against human fungal pathogens *C. albicans* and *C. neoformans*. Some fluconazole-resistant strains of *C. krusei* are also sensitive to the macrotetrolides, while fluconazole-resistant strains of *C. albicans* are most often resistant. Importantly, monactin, dinactin, and trinactin exert potent activity against *C. albicans* biofilms. Dinactin also showed *in vivo* activity against *C. albicans* biofilm. Despite their robust activity against medically important fungi, macrotetrolides were well tolerated by mammals in previous studies [32]. Although our study was small, our results suggest that macrotetrolides have broader and more potent activity against human fungal pathogens than reported previously, and suggest that these compounds may represent promising antifungal drug leads.



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## Supplementary material available online

Supplementary Fig. 1 to be found online at http://www.informahealthcare.com/doi/abs/10.3109/13693786.2012.710917