Journal of Bioscience and Bioengineering
VOL. 121 No. 4, 394–398, 2016

www.elsevier.com/locate/jbiosc

Cryptic antifungal compounds active by synergism with polyene antibiotics

Hiroshi Kinoshita,[1] Mariko Yoshioka,[1] Fumio Ihara,[2] and Takuya Nihira[1,3,*]

International Center for Biotechnology, Osaka University, 2-1 Yamadaoka, Suita, Osaka 565-0871, Japan,[1] National Institute of Fruit Tree Science, 2-1 Fujimoto, Tsukuba, Ibaraki 305-8605, Japan,[2] and MU-OU Collaborative Research Center for Bioscience and Biotechnology, Faculty of Science, Mahidol University, Rama VI Rd., Bangkok 10400, Thailand[3]

Received 1 May 2015; accepted 5 August 2015
Available online 29 August 2015

The majority of antifungal compounds reported so far target the cell wall or cell membrane of fungi, suggesting that other types of antibiotics cannot exert their activity because they cannot penetrate into the cells. Therefore, if the permeability of the cell membrane could be enhanced, many antibiotics might be found to have antifungal activity. We here used the polyene antibiotic nystatin, which binds to ergosterol and forms pores at the cell membrane, to enhance the cellular permeability. In the presence of nystatin, many culture extracts from entomopathogenic fungi displayed antifungal activity. Among all the active extracts, two active components were purified and identified as helvolic acid and terramide A. Because the minimum inhibitory concentration of either compound was reduced four-fold in the presence of nystatin, it can be concluded that this screening method is useful for detecting novel antifungal activity.
© 2015, The Society for Biotechnology, Japan. All rights reserved.

[**Key words:** Antifungal compounds; Polyene antibiotics; Helvolic acid; Terramide A; Entomopathogenic fungi]

Polyene antibiotics are circular molecules that exhibit antimicrobial activity and include 3 to 8 conjugated double bonds in their structure; nystatin and amphotericin B are two examples. Most of the polyene antibiotics were discovered from actinomycetes (1) as fungicides against many eukaryotes, and have been used for medical treatment (2). Polyene antibiotics interact with ergosterol in the fungal cell membrane, make pores, and increase the in- and out-flow of many materials, thereby exerting antifungal activity (3–6). However, when prescribed for humans, these agents often carry serious side effects such as renal dysfunction, electrolyte imbalance, and hypotension, which prevent their further use in medical treatment (2,7). New antibiotics which display high antifungal activity but low toxicity are thus in high demand to deal with the increase of systemic fungal infection.

Despite exhaustive screening of antifungal compounds, the majority of isolated compounds target the biosynthesis and/or function of the fungal cell wall/membrane (8–10), possibly because antifungal compounds targeting components other than the cell wall/membrane have been overlooked due to their difficulty in penetrating the fungal cell wall/membrane and exhibiting their properties.

Based on this assumption, we constructed a bioassay system to discover novel antifungal activities by increasing the cell permeability with polyene antibiotics. We considered that compounds which have been overlooked due to their inability to penetrate the fungal cell/membrane would thus reveal their antifungal activities in the presence of polyene compounds. In this research, we screened antibiotics active against *Candida albicans* (the most common fungal pathogen in humans), which causes systemic infections, especially in susceptible immunocompromised patients (10).

MATERIALS AND METHODS

Strains and media *C. albicans* OUT6266 was used as the indicator strain for bioassay. *C. albicans* was cultivated in Sabouraud medium [glucose 40 g, Bacto peptone (BD Biosciences) 10 g, H_2O up to 1000 ml] at 120 rpm at 28°C for 18 h, and was collected by centrifugation at 3500 rpm ×*g* for 10 min. The cells were washed three times with sterilized H_2O in a volume equal to that of the medium. After adjusting the cell concentration to 2.5×10^7 cell/ml in 20% glycerol solution, the cells were stored at −80°C as the seed culture for bioassay. SMY medium [maltose 4%, Bacto yeast extract (BD Biosciences) 1%, Bacto peptone 1%] and A3M [glucose 0.5%, glycerol 2%, soluble starch 2%, Pharmamedia (Traders Protein) 1.5%, Bacto yeast extract 0.3%, HP-20 (Mitsubishi Chemical Co.) 1.0%] media were used to cultivate entomopathogenic fungi for metabolite analysis. potato dextrose agar [PDA (BD Biosciences) 39 g L^{-1}] was used for the bioassay.

Determination of the polyene concentration used for bioassay Serially diluted solutions of nystatin (Sigma) and amphotericin B (Wako) were prepared with dimethyl sulfoxide (DMSO). Hygromycin B was dissolved with dH_2O and sterilized by filterization. *C. albicans* (1 ml of the stock culture) was inoculated into 250 ml of PDA and spread onto Petri dishes. A series of polyene solutions (200 µl) with or without hygromycin B solution were added to penicillin cups and set on the plates. The inhibition zone was measured after cultivation at 30°C for 24 h.

Preparation of metabolites from entomopathogenic fungi Mycelium stored on a PDA slant at 4°C were inoculated into 5 ml of SMY medium and cultivated at 120 rpm at 28°C for 4–10 days until they grew. The preculture (0.6 ml) was inoculated into 30 ml of SMY or A3M medium in a 100 ml flask and cultivated without shaking at 25°C under light conditions (MLR-352 growth chamber; SANYO). Samples of the culture broth (2 ml) were taken at the 14th day and 21st day, and extracted with 2 ml each of ethyl acetate. The solvent layer was evaporated in vacuo and the residue was used as the extract of the culture supernatant. At the 21st day, the remaining culture broth was extracted with an equal volume of *n*-butanol, and the solvent layer was evaporated in vacuo for use as the extract of the whole broth.

Bioassay in the presence of nystatin An amount of extract equivalent to two ml of culture was dissolved with 390 µl of methanol and used as a metabolite sample. Each sample (190 µl) was added to a paper disc (Φ8 mm) with or without

* Corresponding author at: International Center for Biotechnology, Osaka University, 2-1 Yamadaoka, Suita, Osaka 565-0871, Japan. Tel.: +81 6 6879 7452; fax: +81 6 6879 7454.
E-mail address: nihira@icb.osaka-u.ac.jp (T. Nihira).

1389-1723/$ – see front matter © 2015, The Society for Biotechnology, Japan. All rights reserved.
http://dx.doi.org/10.1016/j.jbiosc.2015.08.003

nystatin (final concentration: 2.9 μg/ml). After drying, the paper disc was put on a plate coated with *C. albicans* and incubated at 30°C for 24 h.

Preparation of metabolites from entomopathogenic fungi cultivated in large scale Preculture (3 ml) prepared by the same procedure for small scale cultivation was inoculated into 150 ml of SMY or A3M medium in 500-ml flasks, and cultivated without shaking at 25°C for 14 or 21 days under light conditions (MLR-352 growth chamber; Sanyo). A 10 ml sample of culture broth was taken on the 14th day and extracted with an equal volume of ethyl acetate. The organic solvent layer was evaporated in vacuo and the residue was used as an extract of the culture supernatant. At the 24th day of cultivation, 10 ml of culture broth was subjected to ethyl acetate extraction in the same manner. The rest of the culture broth was extracted with an equivalent volume of *n*-butanol. The organic solvent layer was evaporated in vacuo and the residue was used as an extract of the whole broth.

Analysis of active components from entomopathogenic fungi *Cordyceps indigotica* HF879 and *Torrubiella minutissima* HF809 were cultivated in A3M medium for 14 days and in SMY medium for 21 days, respectively. After extraction with ethyl acetate and evaporation in vacuo, 975 mg and 240 mg of residues were obtained from 1.5 L culture of *C. indigotica* and *T. minutissima*, respectively. Each extract was analyzed by reverse-phase C18 HPLC [Imtakt Cadenza CD-C18 (Φ4.6 × 75 mm)] with a linear gradient of CH_3CN from 15% to 85% in 0.1% aqueous HCOOH solution (3 min at 15%, 22 min from 15% to 85%, 7 min from 85% to 15%), at a flow rate of 1.2 ml min^{-1}, by monitoring with a photo diode array detector. Samples equivalent to 4 ml of culture broth were loaded. The eluates were collected every 4 min into eight fractions and evaporated in vacuo. The residues were assayed for antifungal activity. The active fractions were further fractionated to determine the active peaks by HPLC using the program described above. The antifungal activity of each distinct peak was also analyzed as described earlier.

Purification of active components The extract from *C. indigotica* HF879 was applied on a Sep-Pak C18 (35 cc) cartridge, and purified by stepwise elution with increasing MeOH concentrations (40 ml of 50%, 20 ml each of 55%, 60%, 65%, 70%, 75%, 80%, 85%, and 90%, and 100 ml of 100% MeOH/H_2O). After evaporation of the 80–95% fractions, 15 mg of the residue was further purified by preparative reverse-phase C18 HPLC with a Capcell Pak C18 column (Shiseido: Φ10 × 250 mm) with isocratic elution using 60% CH_3CN at a flow rate of 4 ml min^{-1}. The peak i, detected at around 18.9 min by UV (230 nm), was collected and evaporated in vacuo to dryness, yielding 5.22 mg.

In a similar manner, the active component from *T. minutissima* HF809 was purified with a Sep-Pak C18 (35 cc) cartridge by stepwise elution with increasing MeOH concentrations (40 ml of 20%, 20 ml each of 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, and 70%, and 100 ml of 100% MeOH/H_2O). After evaporation of the 55–70% fractions, 54 mg of the residue was further purified by preparative reverse-phase C18 HPLC with a Xterra column (Waters: Φ10 × 150 mm) with isocratic elution using 25% CH_3CN at a flow rate of 4 ml min^{-1}. The peak 2, detected at around 7.5 min by UV (210 nm), was collected and evaporated in vacuo to dryness, yielding 2.3 mg.

Purification of helvolic acid from *Metarhizium anisopliae* was carried out according to the previous report (11).

MS was measured by FAB, EI-MS (JEOL MS-700 spectrometer, JEOL Ltd., Tokyo, Japan) and NMR was measured in CD_3OD by a JEOL ECS−400 spectrometer (JEOL Ltd.).

Minimum inhibitory concentration *C. albicans* OUT6266, *Saccharomyces cerevisiae* ATCC9804, *Aspergillus niger* NBRC6341, and *Rhizopus oryzae* NBRC31005 were used as the test strains. The minimum inhibitory concentration was determined by micro-dilution assay (12), while *S. cerevisiae* was cultivated in YPD medium. A synergistic effect was observed by adding target compounds after 1 h of cultivation with nystatin.

RESULTS AND DISCUSSION

Construction of a bioassay system with *C. albicans* as the indicator strain We used *C. albicans* as an indicator strain in the present study; this fungus causes an opportunistic infection, candidiasis, in immunosuppressed patients. First, we determined the optimal concentration of the polyene antibiotics (nystatin and amphotericin B) at which no inhibitory activity against *C. albicans* was detected. Nystatin at a concentration higher than 5.9 μg/ml showed a distinct growth inhibitory area (Table S2). Based on this result, a concentration of 2.9 μg/ml nystatin was chosen for the bioassay. However, the concentration of amphotericin B as the coexisting polyene was not determined because the effective concentration was too low for practical use (<1.5 μg/ml). Subsequently, this assay system was validated using the aminoglycoside antibiotic hygromycin B (HygB), which inhibits protein synthesis inside the cell. Although a very high concentration of HygB (250 μg/ml) was required to inhibit the growth of *C. albicans* in the absence of nystatin, the effective concentration decreased 4-fold in the presence of nystatin (2.9 μg/ml) (Table 1), suggesting that HygB was easily localized intracellularly in the presence of nystatin due to the enhanced permeability of the cell membrane. Therefore, this assay system will assist in the discovery compounds which show slight or undetectable activity with a conventional assay system.

TABLE 1. Minimal inhibitory concentration of hygromycin in the presence of nystatin.

Antibiotics	Hyg concentration (μg/ml)					
	500	250	125	62.5	31.3	15.6
Hyg	++	+	−	−	−	−
Hyg with Nys (2.9 μg/ml)	++	+	+	+	−	−

Degree of anti-*Candida* activity is expressed by the diameter of inhibition zone (d): moderate: 15 >d≧10 mm (++), weak: 10>d≧7 mm (+), no inhibition (−).

Screening for anti-*C. albicans* compounds from the metabolites of entomopathogenic fungi We selected metabolites from entomopathogenic fungi as the source of active compounds against *C. albicans*. Entomopathogenic fungi which infect and proliferate on host insects are thought to produce various bioactive compounds, and indeed, various bioactive compounds have recently been isolated from these fungi (13–18).

Thirty-two representative strains of entomopathogenic fungi (Table S1) from our in-house collection were used for screening. Six different cultivation conditions (with variations in the medium, cultivation time, and extraction method) were used to obtain culture extracts (Materials and methods). Only in the presence of nystatin, 119 out of 192 culture extracts displayed anti-*Candida* activity (Table 2). These results demonstrated that this screening system was useful to detect anti-*Candida* compounds that have been overlooked by conventional assay systems. Subsequently, we further evaluated the strains which displayed strong and/or reproducible activity, to purify and identify the active components.

Ten of the tested strains (Table S3) were chosen and cultivated in large scale to obtain a sufficient amount of active components for structural identification, and it was found that the productivity of active components did not change significantly even in the case of a five-fold scale-up. Among all samples, the active components in the extracts from *Cordyceps indigotica* HF879 and *Torrubiella minutissima* HF809 were selected for further investigation, because they showed the largest inhibitory clear zones in the bioassay.

Purification and identification of the active component from *C. indigotica* To identify the active components in the metabolite of *C. indigotica* among the many peaks detected in HPLC analysis, a bioassay against *C. albicans* was conducted for each fraction obtained by sequential fractionation, with fractions 3, 4, and 5 exhibiting significant activity (Fig. 1A and B). The UV absorption spectra of relatively large peaks (peaks a to i) in these fractions were compared with those registered in Dictionary of Natural Products on DVD (CRC Press). As a result, the peaks a to h in fractions 3, 4 and 5 were suggested to be the members of the indigotide family that were recently discovered from *C. indigotica* (19,20) (Fig. S1, Table S4). To avoid rediscovery of compounds which have already been identified in the same species, we stopped further purification, although the indigotide compounds were not previously shown to have anti-*Candida* activity.

As for fraction 5, we further fractionated the contents, isolated each peak, and evaluated the individual biological activities. Peaks i and h were collected and checked for anti-*Candida* activity (Fig. 1C). Although both peaks possessed anti-*Candida* activity, peak i was further purified to determine its chemical structure because peak i showed a different UV spectrum from those of the indigotide family (Fig. S1). The extract of *C. indigotica* was separated with a Sep-Pak C18 cartridge, and the peak i was present in the fractions eluted

Download English Version:

https://daneshyari.com/en/article/20129

Download Persian Version:

https://daneshyari.com/article/20129

Daneshyari.com