A New Screening Method for Pathway-specific Antifungal Compounds

ATSUKI MORISHITA, SYUJI MURAMATSU, KENTAROU FUKUZAKI, SATORU ISHIKAWA and AKIO MATSUDA

Institute for Life Science Research, Asahi Kasei Pharma Corporation
2-1, Samejima, Fuji-City, Shizuoka 416-8501, Japan

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Recently there has been a dramatic rise in the number of immunocompromised patients within our population, resulting from infection with HIV or from aggressive medical practices. These patients are especially susceptible to infections by opportunistic fungi leading to mycoses. In the course of our antifungal screening program, we have devised an efficient screening method for selective toxicity for fungi. We have taken advantage of recent advances in genomics research and technical advances in high-throughput screening (HTS). This paper describes a screening strategy to detect compounds that inhibit any of the steps in the biosynthetic pathway of fungi. This screening method is rapid, robust, sensitive, and suitable for HTS.

We sought homologous proteins between fungi and mammals, which were essential in fungi. It is appropriate to choose target proteins that have important roles for cell division or biosynthesis of essential cell components. Some antifungal medicines, fluconazole (FCZ), itraconazole (ICZ) and miconazole (MCZ), are notable because they show low toxicity towards humans. They have selective inhibitory activities against biosynthetic enzyme of ergosterol for fungi and cholesterol for humans. We also chose target proteins that participate in biosynthetic pathway of ergosterol. Biosynthesis of cholesterol in humans is the same as that of ergosterol in fungi until the middle of their pathways; biosynthetic enzymes are homologous in each step. Lanosterol 14-alpha demethylase (DM) is a typical enzyme for biosynthesis of ergosterol. In this paper we describe the screening method for selective inhibitors of fungal DM using the engineered strains of yeast.

We prepared three types of strains for this screening. One expressed DM of Candida albicans (S1) and others expressed that each of human (S2) and rat (S3). If there are compounds that have specific inhibitory activity for the fungal enzyme, S1 will not survive in the presence of these compounds but S2 and S3 will survive. So we can easily screen specific activity of compounds by monitoring difference of growth among S1, S2 and S3.

Establishment of Yeast Strains, S1, S2, and S3

Yeast cultures and manipulations were performed by standard methods. Yeast S1 strain was constructed as described below. Candida albicans DM gene was amplified by PCR using Candida albicans genomic DNA as a template. The PCR product was digested by BamHI and Smal, and this fragments were subcloned into BamHI-Smal sites of pYACT3. This plasmid, expressing Candida albicans DM in yeast, was used to transform Saccharomyces cerevisiae YPH500 (Stragene). This transformant was subject to disruption of Saccharomyces cerevisiae ERG11. The disruption was generated by a one-step gene disruption method involving transformation by a linearized fragment of pΔERG11. ERG11 gene was cloned by PCR amplification, and the LEU2 gene from YEp13 (ATCC37115) was inserted into the coding sequence of ERG11, resulting in pΔERG11.

Human DM cDNA and rat DM cDNA were cloned by PCR, using QUICK-Clone cDNA Human Placenta (Clontech) and QUICK-Clone cDNA Rat Liver (Clontech), respectively, as a template. Yeast S2 and S3 strains were constructed by the same method as S1.

Fermentation of Yeast Strains with Test Samples

Medium 1: Bacto-Yeast nitrogen base without amino acids 8.1 g, glucose 24 g, adenine sulfate 20 mg, uracil 24 mg, L-histidine 24 mg, and L-lysine 36 mg were dissolved in 1 liter of distilled water.

Medium 2: Bacto-Yeast nitrogen base without amino acids 9.7 g, glucose 29 g, adenine sulfate 24 mg, uracil 29 mg, L-histidine 29 mg, and L-lysine 43 mg were dissolved in 1 liter of distilled water.

Stock culture: The yeast strain was inoculated into a 15 ml tube containing 3 ml of the medium 1. The culture was incubated for 24 hours at 28°C on a tube shaker. 30% glycerol was added to the culture after incubation. 100 μl of glycerol culture was transferred into 1.5 ml micro tube and

* Corresponding author: morishita.ad@om.asahi-kasei.co.jp
frozen at −80°C.

Seed culture: 50 μl of stock culture was transferred into a 15 ml tube containing 3 ml of the medium 1. The culture was incubated for 24 hours at 28°C on a tube shaker.

Main culture with test samples: The test samples were sterilized in 96-well micro plates before using for assay. When the test sample contained microbial culture broth filtrate, 25 μl of the test sample was mixed with 25 μl of ethanol in a 96-well sample plate. 2 μl/well of sterile sample were dispensed into a 96-well assay plate. When the test sample was from the chemical library, 10 μl of test sample (4 mg/ml in DMSO) was mixed with 200 μl of 50% ethanol in a 96-well sample plate. 2.7 μl/well of sterile sample were dispensed into a 96-well assay plate. After dispensing the test samples into assay plates, the yeast culture was prepared. 10 μl of the seed culture was transferred into 10 ml of the medium 2. 100 μl/well of the culture was transferred into a 96-well assay plate. The plate was incubated for 24 hours at 32°C on a rotary plate shaker.

Scoring Hits
After incubation, the OD at 595 nm was measured (Biorad 550 reader). 50% ethanol was dispensed into control and blank wells. The medium without the seed culture was used for a blank well. OD of control wells reached about 0.4~0.45. Inhibitory activity was calculated as follows:

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\text{Inhibitory activity (\%)} = \frac{(\text{OD of control wells} - \text{OD of sample wells})}{(\text{OD of control wells} - \text{OD of blank wells})} \times 100
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In the first step, inhibitory activity for the S1 strain was screened. If growth inhibitory activity for S1 was higher than 50%, the sample was selected as a first hit. Then activities of hit samples were compared at several concentrations for S1, S2, and S3. If the IC_{50} for S1 was 10 times lower than those for S2 and S3, the sample was designated as a true hit that has selective inhibitory activity against DM of Candida.

Confirmation of Screening Strategy
To confirm the screening strategy, FCZ, ICZ, and MCZ were tested in this assay. Inhibitory activities of these compounds are shown in Fig. 1. They inhibit the growth of S1 selectively.

Screening of Microbial Fermentation Broths and Chemical Library Samples
We have established this screening for 36,000 samples of microbial culture broths and 30,000 samples from a chemical library. There were no samples that showed selective activity for S1 in the microbial culture broths. Only one compound, 5-Chloro-2-phenyl-1H-benzoimidazole (CPBI), from the chemical library showed selective activity. The structure of CPBI is shown in Fig. 2 and the inhibitory activities for S1, S2, and S3 are shown in Table 1. The inhibitory concentration for S1 was 10 times lower than for S2 and S3. The MICs of CPBI, FCZ, and MCZ are shown in Table 2. CPBI has antifungal activity but it also has cell toxicity (data not shown). Antifungal activities have been reported for benzoimidazole.
Therefore, this is the first report describing that CPBI shows the inhibitory activity of DM for its antifungal activity.

The ergosterol biosynthetic pathway is recognized as a good target for selective antifungal compounds. Our screening method is pathway-specific and easy to apply to find inhibitors of enzymes that participate in the ergosterol pathway, for example squalene epoxidase, squalene synthase, and squalene cyclase. We plan to apply this screening method to further targets and other pathogenic fungi.

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References

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