Research Article

Profiling of the effects of antifungal agents on yeast cells based on morphometric analysis

Abraham Abera Gebre, Hiroki Okada, Cholgwang Kim, Karen Kubo, Shinsuke Ohnuki and Yoshikazu Ohya

Department of Integrated Biosciences, Graduate School of Frontier Sciences, The University of Tokyo, Kashiwa, Chiba, 277-8561, Japan

*Corresponding author: Department of Integrated Biosciences, Graduate School of Frontier Sciences, The University of Tokyo, Kashiwa, Chiba, 277-8561, Japan. Tel: +81 (0)4-7136-3650; Fax: +81 (0)4-7136-3651; E-mail: ohya@k.u-tokyo.ac.jp

One sentence summary: Morphometric approach to profile and systematically classify antifungal agents based on their action on yeast.

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ABSTRACT

The incidence of fungal infection and evolution of multidrug resistance have increased the need for new antifungal agents. To gain further insight into the development of antifungal drugs, the phenotypic profiles of currently available antifungal agents of three classes—ergosterol, cell wall and nucleic acid biosynthesis inhibitors—were investigated using yeast morphology as a chemogenomic signature. The comparison of drug-induced morphological changes with the deletion of 4718 non-essential genes not only confirmed the mode of action of the drugs but also revealed an unexpected connection among ergosterol, vacuolar proton-transporting V-type ATPase and cell-wall-targeting drugs. To improve, simplify and accelerate drug development, we developed a systematic classifier that sorts a newly discovered compound into a class with a similar mode of action without any mutant information. Using well-characterized agents as target unknown compounds, this method successfully categorized these compounds into their respective classes. Based on our data, we suggest that morphological profiling can be used to develop novel antifungal drugs.

Keywords: morphological profiling; CalMorph; cell wall; mode of action; target prediction; antifungal agent; systematic classifier

INTRODUCTION

Identification of the molecular targets is indispensable during the development of new antifungal drugs (Hughes et al. 2000; Carrillo-Muñoz et al. 2006; Agarwal et al. 2008). Antifungal drugs with known clear targets can be easily applied for antifungal therapies. Antifungal drugs with distinct molecular targets are attractive because of the effectiveness of their combinatorial usage.

Several potential criteria must be fulfilled by targets of antifungal drugs (Agarwal et al. 2008). A potential target must be essential for fungal cell survival either in the process of static growth, homeostatic stability or infection of the host. It is also important for the target to be a fungal-specific protein; otherwise, even if it is conserved, the affinity of the counterpart to the drugs must be low. Fungal-specific metabolic pathways are attractive targets because each metabolic component is a potential target. Finally, because the plasma membrane can act as a barrier to chemicals, cell surface proteins are preferable as targets.

Due to these restrictions, the intracellular targets of currently available antifungal drugs are limited to the processes related to the cell wall, the cell membrane and ribonucleotide metabolism. Echinocandins such as echinocandin B, caspofungin and micafungin target the biosynthesis of 1,3-β-glucan, a key
fungal cell wall component (Perlin 2007). A recent study of drug-resistant mutant echinocandins suggested that the drugs impact a putative catalytic subunit of 1,3-β-glucan synthase, Fks1p (Johnson, Katiyar and Edlind 2011; Johnson and Edlind 2012). Azole compounds (Bodey 1992) such as fluconazole and micafungin, allylamine compounds (Petryni, Ryder and Stutz 1984) such as terbinafine and naftifine, and morpholine compounds (Polak-Wyss, Lengsfeld and Oesterhelt 1985) such as fenpropimorph and amorolfine impact ergosterol biosynthesis. Azoles, allylamines and morpholines inhibit lanosterol 14α-demethylase, squalene epoxidase and Δ14 reductase/Δ7-Δ8 isomerase, respectively (Carrillo-Muñoz et al. 2006). Ergosterol, a component of the fungal membrane itself, binds to polyene antifungals such as nystatin and amphotericin B (Zygmun and Tavormina 1966). Finally, flucytosine, or 5-fluorocytosine, a fluorinated pyrimidine analog, is a synthetic antifungal drug that inhibits ribonucleotide metabolism (Finland and Schönebeck 1972). Although antifungal drugs with the same structural backbone have similar modes of action (MoA), the reverse is not always the case. New drugs with noble structures could be established without relying on structure-based screening.

Phenotype-based screening has been also recognized as a reliable approach to identifying new antifungal drugs. Such techniques have recently become more powerful after the development of modified phenotypic screens, such as high-throughput, high-content and omics-based screens (Feng et al. 2009; Houle, Govindaraju and Omholt 2010; Roti and Stemmaier 2012; Futamura, Muroi and Osada 2013). Various genomic and genetic tools for the advancement of genome-wide studies in Saccharomyces cerevisiae have been developed (Luesch et al. 2005; Bharucha and Kumar 2007), and a large-scale data set has been deposited in public databases, greatly facilitating the comparison and interpretation of results. Expression profiling and fitness profiling have been widely used for these purposes. It was reported that the pattern of changes in global gene expression can be used as a fingerprint to identify specific pathways perturbed by the chemical compounds (Hughes et al. 2000). Fitness profiling with a decreased or increased gene dosage was shown to facilitate classification of chemical compounds with similar MoAs (Hillenmeyer et al. 2010; Lee et al. 2013).

Another phenotype-based approach that involves the assessment of a high-dimensional cellular response caused by the drugs is image-based profiling (Perlman et al. 2004). We developed the image-based method to infer drug targets based on the yeast morphological changes after treatment (Ohnuki et al. 2010). The morphological profiles induced by the drugs were statistically compared with those induced by deletion of each of 4718 non-essential genes. Based on the ranking of similarity in each mutant, this method was capable of inferring intracellular targets among the 4718 non-essential genes (Ohnuki et al. 2010; Iwaki et al. 2013; Piotrowski et al. 2015). However, no systematic analysis and comparison of antifungal drugs based on morphological profiling has been performed to date.

To gain further insights into antifungal drug profiles, and propose new methods of predicting drug targets without any mutant information, we profiled currently available antifungal drugs using yeast morphology. We analyzed echinocandins (echinocandin B and micafungin), an azole (fluconazole), an allylamine (terbinafine), a morpholine (amorolfine) and a fluorinated pyrimidine analog (5-fluorocytosine). Comparison with deletion mutants of each of 4718 non-essential genes confirmed the MoA of the drugs and revealed unexpected connections among the various cellular processes. We propose that morphological profiling of the drugs can serve as a systematic classifier for a newly discovered compound with similar MoA. Based on our data, we suggest that morphological profiling can be used to develop antifungal drugs.

### MATERIALS AND METHODS

#### Antifungal agents

All analyzed compounds, their acronyms and suppliers are as follows: fluconazole (FCZ; Tokyo Chemical Industry, Tokyo, Japan), terbinafine (TBF; Tokyo Chemical Industry), amorolfine (AMF; Tokyo chemical industry), flucytosine (PCS; Sigma-Aldrich, St Louis, MO, USA), echinocandin B (ECB; a kind gift from O. Kondo, Chugai Pharmaceutical, Tokyo, Japan), micafungin (MCF; Astellas Pharma, Tokyo, Japan), lovastatin (LVS; Wako Pure Chemical Industries, Osaka, Japan), concanamycin A (CMA; Sigma-Aldrich), tunicamycin (TCM; Sigma-Aldrich), bleomycin (BMC; Sigma-Aldrich), hydroxyurea (HXU; Sigma-Aldrich), micafungin (MCZ; Sigma-Aldrich), hygromycin B (HYG; Wako), caffeine (CAF; Nacalai tesque, Kyoto, Japan) and nikkomycin Z (NMZ; Sigma-Aldrich). Stock concentrations and solution preparation methods are summarized in Table 1.

#### Table 1. Chemical compounds used in this study.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Abbreviation</th>
<th>Stock solution</th>
<th>Treatment concentrations</th>
<th>Target cellular process</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluconazole</td>
<td>FCZ</td>
<td>10 mg mL⁻¹ methanol</td>
<td>0, 1,75, 3,5, 5,25, 7 μg mL⁻¹</td>
<td>Ergosterol biosynthesis</td>
</tr>
<tr>
<td>Terbinafine</td>
<td>TBF</td>
<td>10 mg mL⁻¹ methanol</td>
<td>0, 0,0625, 0,125, 0,25, 0,375, 0,5 μg mL⁻¹</td>
<td>Ergosterol biosynthesis</td>
</tr>
<tr>
<td>Amorolfine</td>
<td>AMF</td>
<td>1 mg mL⁻¹ methanol</td>
<td>0, 1,25, 2,5, 3,75, 5 μg mL⁻¹</td>
<td>Ergosterol biosynthesis</td>
</tr>
<tr>
<td>Flucytosine</td>
<td>FCS</td>
<td>0.4 mg mL⁻¹ in distilled water (DW)</td>
<td>0, 0,125, 0,25, 0,375, 0,5 μg mL⁻¹</td>
<td>Nucleic acid biosynthesis</td>
</tr>
<tr>
<td>Micafungin</td>
<td>MCF</td>
<td>10 mg mL⁻¹ in DW</td>
<td>0, 0,10, 15, 20, 25, 30 μg mL⁻¹</td>
<td>Cell wall biogenesis</td>
</tr>
<tr>
<td>Echinocandin</td>
<td>ECB</td>
<td>2 mg mL⁻¹ DMSO</td>
<td>0, 1,2, 3, 4 μg mL⁻¹</td>
<td>Cell wall biogenesis</td>
</tr>
<tr>
<td>Caffeine</td>
<td>CAF</td>
<td>10 mg mL⁻¹ in DW</td>
<td>0, 0,197, 296, 3, 444, 666, 1000 μg mL⁻¹</td>
<td>Cell wall biogenesis</td>
</tr>
<tr>
<td>Nikkomycin Z</td>
<td>NMZ</td>
<td>10 mM in DW</td>
<td>0, 80, 160, 240, 320, 400 μM</td>
<td>Cell wall biogenesis</td>
</tr>
<tr>
<td>Micafungin</td>
<td>MCZ</td>
<td>10 μM in DMSO</td>
<td>0, 2, 4, 6, 8 nM</td>
<td>Ergosterol biosynthesis</td>
</tr>
<tr>
<td>Concanamycin</td>
<td>CMA</td>
<td>100 μM in DMSO</td>
<td>0, 2, 3, 7, 8, 15 nM</td>
<td>V-ATPase</td>
</tr>
<tr>
<td>Lovastatin</td>
<td>LVS</td>
<td>100 μM in DMSO</td>
<td>0, 2, 3, 7, 8, 15 nM</td>
<td>Mevalonate synthesis</td>
</tr>
<tr>
<td>Tunicamycin</td>
<td>TCM</td>
<td>10 mg mL⁻¹ in DMSO</td>
<td>0, 0,125, 2,5, 3,75, 5 μg mL⁻¹</td>
<td>Glycoprotein synthesis</td>
</tr>
<tr>
<td>Hygromycin B</td>
<td>HYG</td>
<td>5 mg mL⁻¹ in DW</td>
<td>0, 0,125, 2,5, 3,75, 5 μg mL⁻¹</td>
<td>Macromolecular biosynthesis</td>
</tr>
<tr>
<td>Hydroxyurea</td>
<td>HXU</td>
<td>10 μg mL⁻¹ in DW</td>
<td>0, 0,125, 2,5, 3,75, 5 μg mL⁻¹</td>
<td>Macromolecular biosynthesis</td>
</tr>
<tr>
<td>Bleomycin</td>
<td>BMC</td>
<td>1 mg mL⁻¹ in DW</td>
<td>0, 0,125, 2,5, 3,75, 5 μg mL⁻¹</td>
<td>Nucleic acid biosynthesis</td>
</tr>
</tbody>
</table>

*Contains 15% (v/v) ethanol and 0.25% NaOH.
Culture conditions for morphological analysis

Yeast culture for morphological analysis was performed as described previously (Ohnuki et al. 2010). The S. cerevisiae haploid strain BY4741 (MATa; his3Δ1 leu2Δ0 met15Δ0 ura3Δ0) was used as the wild-type (WT) strain. Cells were grown at 25°C in yeast rich medium (YPD) containing 1% Bacto yeast extract (BD Biosciences, San Diego, CA, USA), 2% Bactopeptone (BD Biosciences) and 2% glucose (Wako Pure Chemical Industries). Growth inhibitory tests were performed, with or without a compound, at least twice using the biophotorecorder TVS062CA (Advantec, Tokyo, Japan). After calculating the doubling time at several different concentrations of a compound, the concentration that delayed the growth of WT cells by approximately 10% was determined as the linearly interpolated value. Next, a 10% inhibitory concentration was set as the maximum concentration and the three lower concentrations were selected. Cell samples were then grown (n = 5) in the presence of various selected concentrations of the study drugs and WT yeast without any compound treatment (Table 1).

Image acquisition and processing

Morphological data were acquired as described previously (Ohya et al. 2005). We quantified the morphological attributes in budding yeast to obtain 501 parameter values from at least 200 individual cells in each experiment using the automated image-processing software CalMorph (ver. 1.2) for haploid cells. The CalMorph software can be downloaded from SCMC: Saccharymyces cerevisiae Genome Database (Saito et al. 2004). For illustrative purposes, images were processed using Adobe Photoshop CS2 (Adobe Systems, San Jose, CA).

Statistical analysis

Most of the statistical analyses were performed using R ver. 3.0.0 (http://www.r-project.org/). A custom-made Java-based program was used to assess the morphological similarity between the cells treated with each agent and 4718 non-essential gene-deletion mutants, as reported previously (Ohnuki et al. 2010; Ohnuki, Okada and Ohya 2015).

Extraction of morphological features

To elucidate complex phenotypic changes in the cell, we employed prcomp() function, the R package which is used to perform principal component analysis (PCA) on quantitative morphological data as described previously (Ohnuki et al. 2012; Iwaki et al. 2013; Okada et al. 2014; Piotrowski et al. 2015). The dose-dependent parameters were detected by the Jonckheere–Terpstra test. Significant parameters, obtained from five replicated experiments of each drug data set (FDR = 0.05, t-test; Table S1, Supporting Information), were transformed into rank sums to standardize the distribution. To identify morphological features, we executed a PCA on selected parameters using 122 replicated WT morphological data as a null distribution. Consequently, the principal components (PCs), a set of values of linearly uncorrelated variables, are generated. Among several independent PCs extracted, at least one parameter in each PC was selected as a representative parameter (highlighted in yellow, Table S2, Supporting Information) by considering significant absolute loading values (>0.5). Based on the representative parameters, drug-induced morphological changes are depicted.

Linear discriminant analysis

Linear discriminant analysis (LDA) is a machine learning method that generates a classifier based on the combination of variables that best predicts the group to which a given compound belongs. LDA best separates two or more classes (Friedman 1989). To classify and show the difference of the three classes of antifungal agents, we performed LDA as indicated in the following six principal steps.

Step 1: CalMorph analysis. Yeast cells were treated with serial concentrations of antifungal agents and triply stained with fluoresceinisoxycyanate-labeled concanavalin A (FITC-ConA), rhodamine phalloidin and 4’, 6-diadimino-2-phenylindole (DAPI) to obtain fluorescent images of the cell wall, actin cytoskeleton and nuclear DNA, respectively. To describe the cellular responses using quantitative values, fluorescent images were analyzed using CalMorph, which generated quantified 501 morphological parameters for each drug set. The mean values in each parameter were calculated from at least 200 individual cells treated with six different antifungal agents as specific phenotypic descriptors.

Step 2: Performing the generalized linear model (GLM). We applied the GLM, a flexible generalization of ordinary linear regression (Nelder and Wedderburn 1972), to estimate the dose-dependence of the data. The probability distribution functions and the link functions to estimate the effects of a drug on cell morphology in each parameter were used as described in Yang, Ohnuki and Ohya (2014). We subjected the training and test data sets to GLM as follows.

Step 2A: GLM on the training data. For the training data set, effects of six well-known drugs from the three classes were used as a supervisor for the machine learning. The Z value of the Wald test for dose-dependence (a fixed effect of a drug treatment) in each parameter was calculated by simple linear regression with GLM between five or six concentrations of the drug treatment and parameter values (five replications in each concentration). The number of Z values was six for each parameter: three for ergosterol (FCZ, AMF and TBF), one for DNA (FCS) and two for cell wall (MCF and ECB) drugs.

Step 2B: GLM on the test data. Regarding the test data set for cross-validation, Z values of the Wald test for the dose-dependence in each parameter were estimated by simple linear regression with GLM between the drug concentrations and parameter values of three replicates randomly selected from the five replicates of each concentration. The number of Z values calculated in the test data was 1500 for each class (4500 in total) in each parameter: FCZ, AMF and TBF: 500; FCS: 1500; MCF and ECB: 750. Before implementing LDA, Z values of both training and test data set were superimposed onto 122 PCA-rotated replicated WT morphological data sets.

Step 3: Machine learning. At first, one pair of PCs among all pairs of 122 PCs (7381 combinations) was selected for the classifier that can discriminate among drugs with different mechanisms of action. Next, to enhance the efficiency of sorting, a third possible partnering PC was similarly selected from the remaining 120 PCs. Using combinations of these PCs, the classifier was calculated by supervising the machine with a training data set (n = 6).

Step 4: Cross-validation. Validation of the classifier was accomplished by the accuracy estimated from a 3 × 3 contingency table among three categories of the six drugs and predicted categories
RESULTS

Morphological changes induced by antifungal agents

We examined the morphological changes after treating haploid yeast cells with antifungal agents that disrupt three well-known targets—the ergosterol, nucleic acid and cell wall biosynthesis pathways (Table 1). To minimize high-dose side effects, we used the concentration of each drug that delayed the WT cell growth by 10% for morphological analysis (see the section ‘Materials and methods’). We found that cells treated with FCZ, TBF, AMF, FCS, ECB and MCF displayed aberrant morphological phenotypes (Fig. 1A). To explore the dose-dependence of these effects, we quantified 501 morphometric parameters using CalMorph (Ohtani et al. 2004) after treatment with various concentrations of the drugs (Table 1) and analyzed them using the Jonckheere–Terpstra test, a test for an ordered alternative hypothesis within the data sets (Jonckheere 1954). Of the 501 morphological parameters examined, we successfully identified 17, 52, 9, 49, 343 and 251 dose-dependent parameters of FCZ, TBF, AMF, FCS, ECB and MCF, respectively (Table S1A-F; Supporting Information). We did not use nystatin, an agent binding to ergosterol, because it causes lysis of the cells failing to show any morphological changes (Fig. S1, Supporting Information; see Supplemental experimental procedure, and ‘results and discussion’).

We next analyzed these dose-dependent parameters as described previously (Ohnuki et al. 2012). Fig. 1B shows that the FC1 scores increased in a dose-dependent manner for each of the antifungal agents, suggesting that similar morphological changes became progressively more apparent in the data sets. Next, we performed PCA to identify independent features in the dose-dependent parameters (see the section ‘Materials and methods’; Table S2, Supporting Information). Accordingly, we illustrated phenotypic responses to the representative agents and summarized the morphological changes of yeast cells at the indicated cell cycle stages using representative features (Fig. 2A). As shown in Fig. 2B, a moderate relationship was observed between MCF and TBF, but a weak or negligible relationship was recorded between FCS and the other two agents (TBF and MCF). Consistent with their difference in MoA, the agents of the three classes induced distinct alterations in cellular-, actin-, and nuclear-morphology.

Morphological profiling of the antifungal agents—expected similarities

To unravel the MoA of the antifungal agents in more detail, a comparison of the morphological profiles of the cells treated with the agents and those of non-essential deletion mutants with defects in their MoA-related genes was conducted (Ohnuki et al. 2010). Among the 4718 haploid non-essential gene-deletion mutants, we identified morphologically similar gene-deletion mutants (P < 0.05, after Bonferroni correction) after treatment with each drug (Fig. 3; Fig. S2, Supporting Information).

We then analyzed the genes enriched in the mutants similar to those in cells treated with FCS, a nucleic acid biosynthesis inhibitor, using the GO term finder (Boyle et al. 2004). We found that the genes related to maturation of small subunit ribosomal RNA (SSU-rRNA; GO ID: 0000462) were significantly enriched (Fig. 3; Table S4, Supporting Information). FCS is known to be bioconverted into cytostatic fluorouracil, which, in turn, is further converted to metabolites that inhibit fungal RNA and DNA synthesis (Waldorf and Polak 1983; Parker and Cheng 1990; Vermes 2000; Fang, Hoskins and Butler 2004). Therefore, FCS was likely incorporated into newly synthesized RNA and potentially blocked rRNA processing; thus, FCS-treated cells were similar to SSU-rRNA mutants. Treatment with two ergosterol biosynthesis inhibitors (FCZ and TBF) resulted in significant morphological similarities with an erg28 mutant (Fig. 3; Fig. S2, Supporting Information), a mutant endoplasmic reticulum membrane protein, required for ergosterol biosynthesis (Mo, Valachovic and Bard 2004). The correlation coefficients for erg28 were 0.57 and 0.70 for FCZ and TBF, respectively. This finding is consistent with Hughes et al. (2000), who demonstrated by microarray analyses that the ERG28 transcript is induced in erg11, erg2, erg3 and WT cells treated withazole drugs. AMF-treated cells were significantly associated with an erg2 mutant (Fig. S2, Supporting Information), a mutant of C-8 sterol isomerase (Rahier et al. 2008). This result is in line with the findings of Jia et al. (2002) who reported that, like other mopholines, AMF targets primarily Erg2, although it has been shown to also inhibit Erg24 at higher concentrations. The morphology of cells treated with two cell wall biosynthesis inhibitors (ECB and MCF) shared significant similarity with that of two mutants (hoc1 and mnn10), which have defects in ‘cell wall biogenesis’ (Fig. 3, Fig. S2, Supporting Information).

Morphological profiling of the antifungal agents—unexpected similarities

We found some unexpected morphological similarities between the cells treated with ergosterol-affecting agents and vascular proton-transporting V-type ATPase (V-ATPase) mutants. The GO term analysis of mutants similar to cells treated with FCZ, TBF...
Figure 1. Dose-dependence of morphologic changes induced by treatment with antifungal agents. WT cells (BY4741) were cultured until the early log phase at 25°C in YPD medium with or without the indicated concentrations of antifungal drugs. Cells were triply stained with FITC-Con A, rhodamine phalloidin and DAPI to detect the cell wall, actin and nucleus, respectively. (A) Representative images of the cells from five independent experiments are shown. Scale bar, 5 μm. (B) Distribution of PC1 scores. Initially, some dose-dependent parameters of each drug were selected using the Jonkheere–Terpstra test (FDR = 0.05; Table S1, Supporting Information). Morphological data from five replicates were standardized by the rank-sum method, and PC scores that made the greatest contribution to morphological features (PC1s) were plotted versus drug concentration.

and AMF showed no enrichment in the genes responsible for the ergosterol pathway, but the genes responsible for V-ATPases (Table 3; Table S4, Supporting Information). We also observed high morphological similarity between the yeast cells treated with ergosterol-affecting and V-ATPase-inhibiting agents (Fig. S3, Supporting Information). The similarity between the cells treated with ergosterol-affecting agents and vacuolar ATPase-deficient cells suggested a role of ergosterol in V-ATPase function, although there is little indirect evidence (Zhang et al. 2010). We then assessed the effect of ergosterol inhibitors on the vacuole. Cells treated with ergosterol inhibitors showed a decrease in the ergosterol content in WT cells (data not shown). In addition, we observed the reduction of vacuolar quinacrine fluorescence in WT yeast cells (Fig. S4 and Table S5, Supporting Information), implying that the antifungal drugs had impaired vacuolar acidification. Taken together, these data suggest that ergosterol depletion is a likely mechanism of antifungal activity for disrupting V-ATPase function.

Likewise, GO term analysis using mutants that were similar to ECB- and MCF-treated cells showed enrichment in several genes related to vacuolar function (Fig. S2; Supporting Information; Table 3; Table S4, Supporting Information). This unexpected similarity might be because of the cell wall perturbation induced by defective vacuoles. To this end, robust morphological similarity found between inhibitors of cell wall synthesis (ECB and MCF) and the V-ATPase inhibitor (CMA) may support this idea (Fig. S3, Supporting Information). We next tested whether this association could be explained by cell wall defects across those antifungal drugs of different classes (ECB, MCF and CMA). To assess the impact of drug treatments on the
Figure 2. Morphological changes induced by typical antifungal agents of various classes. (A) TBF, FCS and MCF were selected and exemplified as typical agents of each class. Morphological changes induced by the agents were extracted by PCA to identify representative parameters for each agent. Representative parameters used for illustration are summarized in Table S2 (Supporting Information). (B) To investigate the relationships among the representative antifungal agents TBF, FCS and MCF, a correlational network map was constructed. The CORREL() function in Excel was used to calculate the coefficient of correlation among the morphological variables (PC scores) of the two drugs. The 102 PC scores of each agent were estimated by the algorithm used for morphological profiling. Light blue and gray lines and scores near an individual line denote the degree of association: moderate ($R > 0.5$), weak ($R < 0.5$) or negligible ($R < 0.2$) relationship.
cell wall structure, we measured the susceptibility of yeast cells to zymolyase, an enzyme that effectively lyse cell wall of viable yeast cells (Lussier et al. 1997; Ovalle et al. 1998). We found that preincubation of yeast cells with CMA and cell-wall-affecting drugs resulted in an increased sensitivity to zymolyase compared with the mock-treated cells (Fig. S5, Supporting Information). Therefore, we concluded that unexpected similar phenotypes between V-ATPase- and cell-wall-deficient cells are due to the functional connection between these two cellular processes.

Classification of antifungal drugs by MoA

We considered that the comparison between chemical-induced phenotypes and genetic perturbation is a powerful tool to understand the MoA of antifungal agents. However, for this purpose, we needed to obtain mutant information in advance. We report here a new method to classify antifungal agents without any mutant information by performing LDA on quantified morphological data. To that purpose, we summarized our image-profiling strategy into six analytical steps as shown in Fig. 4 (see the section ‘Materials and methods’ for the detailed algorithm). CalMorph analysis was executed on fluorescence microscopic images to obtain quantified morphological data for each drug considered (Step 1). Before applying LDA, we analyzed the multidimensional drug data with GLM (Step 2A), and the resulting figures were designated as specific phenotypic descriptors. The machine supervised with response descriptors of six drug data sets yielded a learned machine that could discriminate drugs according to their MoA (Step 3). Next, GLM was performed on random samples derived from each drug data set (Step 2B). This test data set was used to validate the efficiency of the learning machine (Step 4). Selection of the best classifier was accomplished using the overall cyclic processes of optimization (Step 5). Eventually, the morphological profiling results from the projection of test compounds onto the best classifiers (Step 6) were visualized in 2D phenotypic space.

The developed training algorithm was applied to the high content data of FCZ, TBF, AMF, FCS, ECB and MCF, and the resulting LD scores of the phenotypic responses were depicted on 2D space to visually classify the features of each agent (Fig. 5A). We can see that the first linear discriminant (LD1) separated the classes quite well, but the second linear discriminant (LD2) added less valuable information. In the phenotypic space, the color-filled circles represent the distribution of the six drugs from the three classes; solid arrows represent the direction of training data separation by the LDA. Therein, drugs that have similar activities formed a cluster at a specific distance from the center. Cross-validation of the test set showed clustering of
Figure 4. Schematic illustration of an image-based antifungal agent-profiling method. The image-based antifungal drug-profiling technique involves six principal steps, as described in the sections ‘Results’ and ‘Materials and Methods’. Images from A-I denote the following: (A) dose-dependent antifungal agent treatment of yeast cells; (B) CalMorph-processed morphological data of triply stained cells; (C) training data set represented in six different colors; (D) test data set generated from random samples; (E) output of machine learning; (F) result of cross-validation; (G) optimum systematic classifier; (H) test compound to be profiled by the best classifier; (I) the results of target prediction.
distribution. The results suggested that the classification of antifungal agents based on the morphological profiles of the three classes reflects their intracellular MoA.

**Classification of other antifungal agents**

In our machine learning approach, we anticipate that when the test agents form a group with a particular training set compound, they are likely to have similar cellular targets. To evaluate our proposed method, we selected a well-characterized drug set containing 10 antifungal agents as 'target unknown' drugs data (Table 2). The profiles of phenotypic multiparameters of these test compounds were visualized in 2D scatter plot. The possible target drug class to which a given compound of interest may belong was determined based on their posterior probability scores. Intriguingly, an optimized classifier assigned nearly all of the agents into the three categories with the highest posterior probability scores > 0.90 (Fig. 5B), indicating that well-characterized drugs were classified by MoA.

**Classification of other antifungal agents: cell-wall-affecting drugs**

Of the bioactive test compounds, our image-profiling method suggested that CAF and NMZ were significantly associated with a cluster of distinguished cell-wall-disrupting drugs, ECB and MCF (Fig. 5B). Essentially, both CAF and NMZ are known to affect the yeast cell wall. CAF is an analog of purine bases that interferes with biogenesis of the cell wall in the budding yeast (Levin 2005; Kuranda et al. 2006), which is crucial to maintain cell shape and integrity (Klis 1994). CAF activates the cell wall integrity signaling by triggering the Rlm1 transcription factor via phosphorylation by Mpk1, mitogen-activated protein kinase pathway (Watanabe et al. 1997; Kuranda et al. 2006; Truman, Kim and Levin 2009). NMZ prevents chitin synthesis in the fungi cell wall by acting as a competitive inhibitor of UDP-N-acetylglucosamine, a substrate for chitin synthase (Gaughran et al. 1994). Thus, our machine learning method was successful in profiling cell-wall-affecting drugs based on their specific phenotypic response.

**Classification of other antifungal agents: nucleic-acid-acting drugs**

Some of the nucleic-acid-synthesis-interfering compounds—such as HXU, BMC, TCM and HYG—were classified along with FCS, an inhibitor of fungal DNA and RNA synthesis and protein translation (Waldorf and Polak 1983) (Fig. 5B). HXU is an antitumor agent with antileukemic activity, causing the inhibition of ribonucleotide reductase activity and consequent suppression of DNA synthesis (Chang and Cheng 1978). BMC binds to DNA and causes single- and double-strand breaks catalytically, resulting in inhibition of DNA biosynthesis (Chen and Stubbe 1983). HYG is an aminoglycosidic agent that inhibits protein synthesis by disrupting translocation and promoting mistranslation at the 80S ribosome (Ahmad et al. 1980). This similarity in their MoAs might have induced similar morphological profiles.

**Classification of other antifungal agents: ergosterol-affecting drugs**

MCZ and AF, which interfere with the fungal sterol synthetic pathway (Shah Alam Bhuiyan et al. 2007; Isham and Ghannoum 2010), were classified into the cluster of ergosterol synthesis inhibitors (Fig. 5B; Fig. S6, Supporting Information). MCZ inhibits ergosterol biosynthesis at the step of sterol 14α-demethylation in the ergosterol biosynthetic pathway (Ghannoum and Rice 1999). However, AF blocks the same pathway at the step of nearly all of the test data points around the six points of the training data set (Fig. 5A). In this figure, the diamond symbols denote median points of the test data, and the dashed arrows show the directions of the test data set separation. Even if the distance of the median points of the training and test sets from the center are different, the direction is similar. In this space, specific phenotypic responses were shown as a distinct cluster

![Figure 5. Classification of antifungal drugs based on their difference in morphological profiles in 2D phenotypic space. (A) The clustering pattern of the test data set around six data points of the training set from antifungal agents of three distinct classes. Clusters represent the test data distribution of the cell wall (red, left side), ergosterol (green, middle) and nucleic acid (black, right side) acting drugs, respectively. Training data (filled circles); test data median points for each drug class (diamond symbol). The positions of the median points for the test data (dashed arrow), and training data (solid arrow) from the center (green open circle; average of posterior probability scores) are indicated. (B) Target profiling of test antifungal compounds. A biplot shows classification of the test compounds into different classes with similar mechanisms of action by the optimum classifier. LD1 and LD2: linear discriminant functions 1 and 2; larger circle (test data distribution), small circles (exact position of each test compound), small filled circle (correctly classified) and small open circle (incorrectly classified).](image-url)
Table 2. Comparison of predictions of target-unknown drug classes by LDA analysis versus experimental research evidence.

<table>
<thead>
<tr>
<th>Drug name</th>
<th>Abbreviation</th>
<th>Predicted drug class</th>
<th>Target, mechanism of action and/or function</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caffeine</td>
<td>CAF</td>
<td>Cell wall</td>
<td>Blocks cell wall biosynthesis</td>
<td>Levin (2005); Kuranda et al. (2006)</td>
</tr>
<tr>
<td>Nikkomycin Z</td>
<td>NMZ</td>
<td>Cell wall</td>
<td>Inhibits chitin synthase of S. cerevisiae</td>
<td>Gaughran et al. (1994)</td>
</tr>
<tr>
<td>Miconazole</td>
<td>MCZ</td>
<td>Ergosterol</td>
<td>Blocks the biosynthesis of ergosterol, inhibits sterol 14α-demethylase</td>
<td>Ghannoum and Rice (1999)</td>
</tr>
<tr>
<td>Amorolfine</td>
<td>AMK</td>
<td>Ergosterol</td>
<td>Inhibits Δ14 reductase and Δ7-Δ8 isomerase</td>
<td>Shah Alam Bhuiyan et al. (2007)</td>
</tr>
<tr>
<td>Lovastatin</td>
<td>LVS</td>
<td>Ergosterol/Nucleic acid</td>
<td>HMG-CoA synthase inhibitor; disrupts the cholesterol synthesis pathway; Mediates G1 arrest via inhibition of proteasome</td>
<td>Alberts et al. (1980); Rao et al. (1999)</td>
</tr>
<tr>
<td>Tunicamycin</td>
<td>TCM</td>
<td>Nucleic acid</td>
<td>Affects bud emergence and initiation of DNA synthesis; Inhibitor of yeast glycoprotein synthesis</td>
<td>Kuo and Lampen (1974); Vai, Popolo and Alberghina (1987)</td>
</tr>
<tr>
<td>Hygromycin B</td>
<td>HYG</td>
<td>Nucleic acid</td>
<td>Inhibiting protein synthesis; Stabilizes the tRNA-ribosomal acceptor site, thereby inhibiting translocation</td>
<td>Borovinskaya et al. (2008); Shoji, Walker and Fredrick (2009)</td>
</tr>
<tr>
<td>Hydroxyurea</td>
<td>HXU</td>
<td>Nucleic acid</td>
<td>Inhibits DNA synthesis; Reduces mRNA levels of small subunit of ribonucleotide reductase (RNR2)</td>
<td>Timson (1975); Moore and Hurlbert (1985)</td>
</tr>
<tr>
<td>Bleomycin</td>
<td>RM C</td>
<td>Nucleic acid</td>
<td>DNA fragmenting agent</td>
<td>Chen and Stubbe (2005)</td>
</tr>
<tr>
<td>Concanamycin A</td>
<td>CMA</td>
<td>Nucleic acid</td>
<td>Inhibits vacuolar-type H+ -ATPases</td>
<td>Dröse et al. (1993)</td>
</tr>
</tbody>
</table>

*AMK—Amorolfine data acquired in different experiment.

Table 3. Enrichment of vacuolar acidification defects from WT cells treated with ergosterol and cell-wall-affecting drugs.

<table>
<thead>
<tr>
<th>Agent</th>
<th>GO category</th>
<th>GO term</th>
<th>P-value</th>
<th>Annotated genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>FCZ</td>
<td>Process</td>
<td>Vacular acidification</td>
<td>1.31E-07</td>
<td>VMA1, VMA3, VMA16, VMA5, VPH2, VMA6, VMA4, VMA1</td>
</tr>
<tr>
<td>TBF</td>
<td>Process</td>
<td>Vacular acidification</td>
<td>3.12E-07</td>
<td>VMA2, VMA1, VPS3, VMA3, VMA7, VMA16, VMA5, VPH2, VMA6, VMA4, VMA11</td>
</tr>
<tr>
<td>AMF</td>
<td>Process</td>
<td>Vacular acidification</td>
<td>8.41E-08</td>
<td>VMA2, VMA1, RRG1, RAV2, VMA3, VMA8, VMA7, VMA5, VPH2, MEH1, VMA6, VMA4, VMA11</td>
</tr>
<tr>
<td>ECB</td>
<td>Process</td>
<td>Vacular acidification</td>
<td>6.10E-06</td>
<td>VMA2, VMA3, VMA7, VMA16, RAV1, VMA5, VPH2, VMA6, VMA4, VMA11, VMA13</td>
</tr>
<tr>
<td>MCF</td>
<td>Function</td>
<td>H+ -transmembrane transporter activity</td>
<td>0.00264</td>
<td>VMA3, VMA5, VMA6, ATP18, VMA4, ATP4, VMA11, ATP15</td>
</tr>
</tbody>
</table>

Δ14 reductase and Δ7-Δ8 isomerase (Shah Alam Bhuiyan et al. 2007). Therefore, it is conceivable that the data of these agents were plotted at or nearby the cluster organized by the ergosterol biosynthesis inhibitors (Fig. 5B). LVS is another drug that inhibits ergosterol biosynthesis (Fig. S6, Supporting Information). Independent of its hydroxymethyl glutaryl-CoA reductase inhibition (Alberts et al. 1980), LVS reduces proteasome activity, leading to G1 phase arrest (Rao et al. 1999). The newly established method plotted LVS in the space between the clusters of ergosterol- and nucleic-acid-synthesis-interfering agents (Fig. 5B), indicating that the high-content system was successful in profiling phenotypes by drug function.

**DISCUSSION**

We analyzed the image-based morphological profiling of the currently available antifungal agents and developed a new profiling method that facilitates the prediction of drug targets. Our system used quantified multiparametric data based on morphological alterations induced by a candidate drug. Most of the drugs were classified into the three training set groups, indicating that the system can successfully recognize the morphological changes of a drug and group them into drugs with similar MoAs. The designed method identifies the biological targets of a compound of interest without relying on any mutant information in advance. In the current situation in which we only have a reference dataset for non-essential mutants, this approach can also be used for the prediction of essential gene targets of a certain compound. A similar approach can be applicable to higher eukaryotes once the system is established in the budding yeast, a proven model organism for studying related human diseases. Thus, our results propose the potential use of this new profiling method as an interesting powerful tool to predict the target of a small molecule of interest.
Comparison with the fitness profiling approach

The multimorphic method employed 501 morphologic parameters to predict drug targets. A comparable method, such as fitness profiling, which employs growth as a primary phenotypic descriptor, has been developed in yeast chemical genomic studies. The fitness approach identifies the likely target of a given compound in two discrete assays—haploinsufficient profiling (HIP) and homozygous profiling (HOP). In the HIP assay, essential gene targets of a certain compound of interest could be identified from the fitness data of the heterozygote strain deleted for the drug target (Giaever et al. 1999; Lum et al. 2004; Lee et al. 2005). In HOP, growth data from a complete loss-of-function deletion in a diploid strain allow identification of non-essential gene targets of a compound (Parsons et al. 2004, 2006; Dudley et al. 2005; Fry, Begley and Samson 2005). Unlike fitness profiling, our profiling system facilitates genome-wide target assessment from multidimensional cellular responses caused by the drugs in a systematic manner once the training database is sufficiently diverse. Therefore, morphological profiling and fitness profiling are complementary, and serve as new drug-prediction tools.

Comparison with other image-profiling approaches

Our chemical genomics research wing focuses on developing various methods for drug target prediction based either on the assumption that chemicals should phenocopy the mutation or screening drugs that share similar perturbation profiles. We previously reported a method of exploring the cellular functions affected by candidate compounds by comparing with the phenotypes of yeast non-essential gene-deletion mutants (Ohnuki et al. 2010). Therein, several functionally related genes, and potentially affected cellular pathways, were identified in addition to previously known target genes of studied compounds. This approach was limited in its ability to cross-examine all relevant gene targets because it interrogates only non-essential gene targets; no information related to essential genes could consequently be inferred. The new method developed in this study was a complementary approach, enabling us to extrapolate a drug target and other pertinent information without any reliance on a mutant database. The machine learning technique sorts a new compound into a class of drug with a similar MoA, defining the MoA of the compound from different viewpoints. Remarkably, the combinatorial usage of these two image-profiling methods has allowed us to leverage the scale of our study by not only identifying candidates of drug targets but also mapping detailed phenotypic information on the cellular response to any conditions that may induce morphologic changes.

Application of linear classification models for drug discovery

In this study, we developed the method that learns phenotypic profiles induced by the drugs of a training set and classifies bioactive substances. Our profiling system involves a simple procedure involving triple staining of the cells (the cell wall, DNA and actin), extracting high-dimensional phenotypic data using CalMorph, and sorting compounds into a class with a similar MoA. Another chemical-genetic phenotype-profiling approach, morphobase (Futamura et al. 2012), was developed using the images of mammalian cells. Morphobase strategy engages PCA and similarity search software to detect targets of a candidate small molecule. Our expedient image-profiling tool employs LDA, which performs dimensionality reduction while preserving as much of the class discriminatory information as possible, attempting to explicitly model the differences among the classes of data (Swets 1996; Martinez and Kak 2001; James 2012). LDA best discriminates up to three classes, yet can achieve average classification accuracies of above 80% for a number of classes until four (Castro 2012). To create a quantitative structure–activity relationship model and identify new compounds from molecular topology databases, other studies used LDA and multiple linear regression (Gozalbes et al. 2000; Mahmoudi et al. 2006, 2008). Their model could discriminate between inactive and active compounds by learning the drug's structural patterns. This approach and our method are both powerful in target prediction and deduced biological information despite the difference in screening criteria (e.g. structure vs phenotype) used and number of features involved (Gozalbes et al. 2000; Ohya et al. 2005).

Considering the MoA, a certain compound was unexpectedly assigned to a different antifungal agent’s class by the classifier. We expected, for example, that CMA, a potent V-ATPase inhibitor (Dröse et al. 1993), to be classified into the cell wall synthesis inhibitor group. However, it was ascribed notably to a different drug group. This might reflect the limitation of such linear methods as LDA. To increase its discrimination power, non-linear multiclass models such as neural networks can be used as a supervised classification technique (Terfloth and Gasteiger 2001; Concu et al. 2010). Our profiling technique is a quantitative morphology-based system that attempts for the first time to use LDA for drug target prediction starting with a small training data set. Expanding the compound database of diverse therapeutic categories could enhance the effectiveness of this image-profiling technology.

Morphological similarities among the antifungal agent-treated cells

Our quantitative morphological analyses showed distinct but somewhat similar morphological phenotypes among the cells treated with ergosterol-,- vacuole- and cell-wall-acting agents. It is important to know the mechanism of their similarity because it is directly related to the understanding of the fungal physiological system. Experimental evidence, in fact, has supported their functional relationships; ergosterol-deficient cells showed no acidification of vacuoles as ergosterol is critically required for V-ATPase activity; and vacuolar-deficient cells showed cell wall defects, suggesting the role of the V-ATPase in fungal physiology is wide ranging to influence the cell wall biosynthesis pathway probably via its effect on the secretory vesicles transport to the fungal cell tip. These findings are also of particular interest from therapeutic aspects because of the frequent combinational use of antifungal drugs in therapy. It is known that a synergetic antifungal effect of 11% was recorded for the antifungal combination of MCF with FCZ against clinical isolates Candida spp. without any antagonistic influence (Nishi et al. 2009). AMF also showed a synergetic combination with TBF and azole drugs (Polak-Wyss 1995). These facts suggest that the high-content profiling of antifungal drugs is useful for the understanding of yeast cell biology and pharmacology of antifungal agents.

CONCLUSION

To facilitate drug discovery, methods for elucidating the potential mechanisms of action and identifying cellular targets of a candidate antifungal compound are needed. As an alternative
to the current phenotypic profiling approach, we developed a new method that predicts a drug target independent of its previous background and without any mutant information. This image-profiling tool successfully classified well-characterized compounds into their specific classes based on their MoA, hence facilitating the prediction of compounds with unknown mechanisms. The profiles of clinically available antifungal drugs were determined by comparing the phenotypic profiles between a gene mutation reference set and a compound of interest, which provided possible unreported molecular targets. Additionally, our multidimensional analysis uncovered an unexpected relationship among ergosterol, V-ATPase and cell-wall-targeting drugs.

**SUPPLEMENTARY DATA**

Supplementary data is available at FEMSYR online.

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