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The global effect of exposing bakers' yeast to 5-fluoruracil and nystatin; a view to Toxichip

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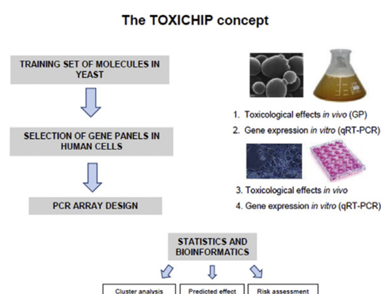
HIGHLIGHTS

- The expression profile of selected genes was analyzed by qRT-PCR.
- A system biology analysis helped to identify molecular targets of 5-FU and nystatin.
- Biomarkers for health or environmental risk assessment were identified.

GRAPHICAL ABSTRACT



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ABSTRACT

A genome-wide screen of a haploid deletion library of bakers' yeast (*Saccharomyces cerevisiae*) was conducted to document the phenotypic and transcriptional impact of exposure to each of the two pharmaceutical products 5-fluorouracil (an anti-tumor agent) and nystatin (an anti-fungal agent). The combined data set was handled by applying a systems biology perspective. A Gene Ontology analysis identified functional categories previously characterized as likely targets for both compounds. Induced transcription profiles were well correlated in yeast and human HepG2 cells. The identified molecular targets for both compounds were used to suggest a small set of human orthologues as appropriate for testing on human material. The yeast system developed here (denoted "Toxichip") has likely utility for identifying biomarkers relevant for health and environmental risk assessment applications required as part of the development process for novel pharmaceuticals.

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1. Introduction

The development of pharmaceuticals, food additives and cosmetics relies heavily on toxicological tests being performed on animal models, but there is an increasing pressure to replace animal experimentation by *in vitro* animal cell lines, supported by transcriptional profiling (Regulation EC No. 1907/2006). The concept of toxicogenomics assumes that the set of toxic effects induced by exposure to a given compound is accompanied by changes in gene expression, the identity of which is revealing with

Abbreviations: 5-FU, 5-fluorouracil; ATCC, American Type Culture Collection; DMEM, Dulbecco's Modified Eagle Medium; G418, Geneticin; GO, Gene ontology; GP, Growth Phenotype; IC_{50}, half-maximal inhibitory concentration; KEGG, Kyoto Encyclopedia of Genes and Genomes; MIC_{99}, Minimum inhibitory concentration; PBS, Phosphate buffered saline; PCA, Principal Component Analysis; qRT-PCR, reverse transcriptase quantitative PCR; YPD, Yeast extract, bactopeptone, dextrose.

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respect to the mechanistic basis of the toxicity (Cui and Paules, 2010). Such tests commonly utilize a DNA microarray platform, versions of which are available based on sequences derived from nematode (*Caenorhabditis elegans*), frog (*Xenopus laevis*), fish (*Danio rerio*), rodent (rat and mouse) and primate (including human) (Qi et al., 2011). Bakers' yeast (*Saccharomyces cerevisiae*) has been widely used as a eukaryotic model to investigate biological, physiological and pathological processes, and has some traction in the area of toxicogenomics (Gaytán and Vulpe, 2014), largely because of its exhaustive genomic, transcriptomic and proteomic datasets, but also because of the prior development of a genome-wide set of heterozygous-homozygous diploid and haploid deletion mutants (Giaever and Nislow, 2014), and because its rapid cell division process speeds the throughput of the test (dos Santos et al., 2012; dos Santos and Sà-Correia, 2015). The yeast proteome harbors orthologs for 46% of human proteins, spanning a wide variety of function (Venter et al., 2001). Yeast mutants have proven highly informative in revealing the identity of genes either required for, or inimical to growth in the presence of a given challenge (Roemer et al., 2012; Venancio et al., 2012).

The current paper describes the exploitation of a combined *in vivo* and *in vitro* yeast data set to develop an array of genes, selected on the basis of their known or demonstrated involvement in the cellular response to certain pharmacologically active compounds. Here, as a validation exercise, two compounds were selected, namely 5-fluorouracil (5-FU) and nystatin, both of which have toxic effects both on the patient and more generally in the environment. The pyrimidine analog 5-FU is used to treat certain solid tumors (Longley et al., 2003), while nystatin, a polyene antibiotic derived from Streptomyces, is widely administered to control a range of fungal infections. It causes the formation of pores in the fungal membranes (Meyer, 1992) which allow for the uptake of large molecules not normally taken up by yeast (Marmiroli et al., 1985).

2. Materials and methods

2.1. Yeast strain and culture conditions

A representation of the yeast homozygous diploid and haploid deletion strains (4634 lines) was purchased from Open Biosystems (Huntsville, AL, USA). Most of these cell lines have been established in a MATα BY4742 background, but a subset of 151 strains was created in a MATα BY4739 background. All cells were grown overnight at 30 °C on YPD medium (10 g L^{-1} yeast extract, 20 g L^{-1}peptone, 20 g L^{-1}dextrose, obtained from Sigma–Aldrich Corp., St. Louis, MO, USA). The reaction of strains identified as being either more sensitive or more tolerant than the wild type strain (WT) to either 5-FU or nystatin (both compounds obtained from Sigma–Aldrich Corp.) was validated using a spot assay, in which a 10X serial dilution (10^1–10^6 cells/mL) was made up in YPD and a 4 μL aliquot of each dilution was spotted on YPD-agar (2% agar) plates, in the presence or absence of either 50 μM 5-FU or 0.7 μM nystatin, after which the cells were cultured at 30 °C.

2.2. The determination of half maximal (IC_{50}) and minimal (MIC_{99}) inhibitory concentrations

A serial dilution spot assay was carried out using the WT strain BY4742 to determine both the IC_{50} and the MIC_{99} for both 5-FU and nystatin.

The range of concentrations tested for the former parameter was, respectively, 10–1000 μM and 0.01–2.00 μM, and for the latter 10–1000 μM and 0.5–5.0 μM. The IC_{50}s were calculated using the Sperman-Karber method (Hamilton et al., 1977), and were 50 μM (5-FU) and 0.7 μM (nystatin), while the MIC_{99}s were, respectively, 750 μM and 4 μM.

2.3. Screening the yeast mutants for response to 5-FU and nystatin exposure

The phenotypic screen was carried out following the protocols described by Ruotolo et al. (2008). Each line was seeded into 150 μL liquid YPD containing 200 μg mL^{-1} G418 (Sigma–Aldrich Corp.) using a 384 pin tool (Nalgene Nunc International Corp., Rochester, NY, USA). After holding at 30 °C for 48 h with shaking, the cells were transferred via a library copier (Open Biosystems) to YPD-agar lacking G418, supplemented with the IC_{50} for either 5-FU or nystatin. After holding for 72 h at 30°C, the growth of each cell line was quantified by measuring signal intensity relatively to the background signal, using a Quantity One Gel Doc system device (Bio-Rad Laboratories, Inc., Hercules, CA, USA). A mean "growth intensity" was then calculated for each mutant, and this was normalized by dividing by the mean growth intensity of the control WT strain to produce a "growth phenotype" (GP). Based on the mean of five replicates, each mutant was allocated to one of three categories: similar to WT (GP in the range 0.5–1.5), sensitive ($GP < 0.5$) or tolerant ($GP > 1.5$). This method was preferred to DNA microarray or PCR analysis (Giaever et al., 2002) because it does not suffer of biases linked to original sampling of the DNAs.

2.4. RNA isolation and quantitative real time PCR (qRT-PCR)

The parental WT strain MATα BY4742 was seeded into 10 mL liquid YPD containing the IC_{50} for either 5-FU or nystatin and incubated at 30 °C for 18 h, after which RNA was extracted using a RNeasy® Midi kit (Qiagen GmbH, Hilden, Germany), following the manufacturer's protocol. The concentration of RNA recovered was estimated spectrophotometrically (Varian Cary 50, Agilent Technologies, Santa Clara, CA, USA). A 400 ng aliquot of each RNA sample was reverse-transcribed using a Quantitect® Reverse Transcription kit (Qiagen), following the manufacturer's protocol. The subsequent qRT-PCR was based on 5 ng cDNA template (replicated three time), 2X Power SYBR® Green PCR (Life Technologies, Carlsbad, CA, USA) and 250 nM of each primer pair (sequences detailed in Tables S1–S3). The reactions were conducted in a 7900HT Fast Real-Time PCR System device (Life Technologies), applying the following thermal regime: 50°C/2 min, 95°C/10 min, then 40 cycles of 95°C/15 s, 60°C/60 s. The subsequent melting curve analysis applied immediately after the amplification procedure, increasing the temperature from 60 °C to 95 °C at a rate of 0.2 °C s^{-1}. All qRT-PCR data were analyzed using the 2-ΔΔCT method (Livak and Schmittgen, 2001), as implemented in the Data Assist v2.0 software package (Life Technologies). The optimal reference genes, selected on the basis of geNorm v 3.4 (included within Data Assist v2.0), were *UBC5* for the 5-FU experiment and *ARP7* for the nystatin data set. Each qRT-PCR data point represented the mean of three technical replicates.

2.5. Statistics, bioinformatics and system biology analysis

All statistical analyses were performed using routines implemented in SPSS 21.0.0 for Windows (© Copyright IBM Corporation 1989, 2012). The Kolmogorov–Smirnov non-parametric test was applied to compare the mutant GPs with that of WT. The Spearman Rho non-parametric correlation test was used to correlate the level of transcription of specific genes and the effect on the GP of its deletion. Principal Component Analysis (PCA) was performed using SPSS 21.0.0. The identity of the deleted gene in each mutants exhibiting a non-WT GP was established by reference to Saccharomyces Genome Database (http://www.yeastgenome.org).

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