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## Screening technologies

# Yeast as a screening tool

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The versatile genetic malleability of yeast, and the high degree of conservation between its cellular processes and those of human cells, have made it the model of choice for pioneering research in molecular and cell biology over the past four decades. These characteristics of yeast, taken together with technical advantages such as simple growth conditions, rapid cell division and the development of a wealth of genetic tools for analysis of biological functions, have expanded the application of yeast as screening tool to the field of drug discovery.

## Introduction

The rapid progress in elucidating the mechanisms underlying human diseases, taken together with the enormous amount of data that has been generated by the genome-wide analysis of biological functions, are significantly increasing the number of proteins that could be targeted by drugs for potential therapeutic treatment (for a review, see [1]). Parallel to these developments, the number and diversity of compounds that can be tested for activity against these targets is also rapidly expanding. The combination of these trends has spurred the development of increasingly rapid, selective and reliable screening assays to be applied in the early phase of drug discovery. Most of the assay formats developed to date can be grouped into biochemical (*in vitro*) or cell-based assays. This article describes the applicability of yeast as a model system and a screening tool for cellular assays in the early phase of drug discovery.

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## Biochemical and cellular assays for drug discovery

Automated high-throughput screens (HTS) to select inhibitors of potential therapeutic targets from large compound collections have originally been developed with biochemical assays. Indeed, many types of cell-free assays, which had been initially developed to measure in a controlled fashion the activity of purified proteins, could be readily converted to HTS by applying detection systems that do not require separation of the reaction product from the substrate. In the past few years, cell-based assays using engineered mammalian cells and microorganisms have become an increasingly attractive alternative to *in vitro* biochemical assays for HTS in the early phase of the drug discovery process. The requirements for such cell-based (*in vivo*) assays are the ability to examine a specific cellular process triggered by a defined target and a means to readily measure its output in a HTS system. The availability of an increasing number of biotechnological tools to genetically modify cells and microorganisms has allowed the development of simple read-out assays for cellular processes, some of which can be applied to automated systems in HTS [2–6]. Cell-based assays have noteworthy advantages over *in vitro* assays. First, cell-based assays do not require purification of the target protein and therefore eliminate investment of resources to gain the necessary knowledge for obtaining a biochemically active target (this advantage has become particularly important with the increasing number of proteins that could be potentially targeted for drug development). Second, the activity of the target protein, as well as the potential effects of compounds, occur in a cellular context that recapitulates the natural

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physiological state more closely than *in vitro* assays. Third, cell-based assays can immediately select against compounds that are generally cytotoxic and can provide information on cell permeability, stability, and solubility of the inhibitor. Thus, so-called 'hit' and 'lead' compounds that are identified through cell-based assays have already passed important validation steps for a combination of properties that make a successful drug candidate. The availability of this information provides a head start compared to many *in vitro* assays and can save valuable time and costs in the development of the drug.

### Yeast as a model system for drug discovery

Cellular screens should ideally be performed with cells of human origin, which evidently provide the most physiologically relevant model system. However, human cells are expensive to culture and sometimes difficult to propagate in automated systems used for HTS. Moreover, the effects of redundant processes on the measured output can be difficult to control and to distinguish from the effects that are expected to be specific for the defined target; and genetic manipulation of mammalian cells is generally problematic and time-consuming. Yeast genetic systems have emerged as powerful tools for measuring the activity of proteins and for detecting and characterizing molecular interactions [7]. In yeast cells, the function of human proteins can often be reconstituted and aspects of some human physiological processes can be recapitulated because of the high degree of conservation of basic molecular and cellular mechanisms between yeast and human cells [5–10]. Thus, yeast represents an inexpensive and simple alternative system to mammalian culture cells for the analysis of drug targets and for the screening of compounds in a heterologous, yet cellular, eukaryotic environment.

One major advantage of yeast over mammalian cells is provided by the versatile genetic malleability of this organism [11]. This characteristic of yeast, as well as the long scientific experience in yeast genetics and molecular biology, has allowed the development of a wealth of experimental tools and genetic selection systems that can now be readily converted to HTS formats for drug discovery. These molecular and cellular tools include advanced plasmid systems, homologous recombination techniques and readily scored markers, including the easy application of conditional growth selection systems. Moreover, in contrast to mammalian cells, where redundant processes often interfere with unambiguous measurement of a specific effect on a given target, a heterologous yeast system permits a clean read-out in a null-background environment for the expression of the human protein. Finally, although yeasts are surrounded by a cell wall and their plasma membrane composition differs to some extent from that of their mammalian counterparts, it is now known that the cell wall itself does not limit permeability of

small molecules, and that compound efflux rather than membrane penetration is the mechanism that mostly contributes to relatively low intracellular concentrations of compounds. In yeast, this process can be readily controlled, and its effect dramatically reduced, by deleting the major ABC transporters [12].

### Yeast as a screening tool: case studies

The possibility of testing human protein function in the cellular environment of a heterologous eukaryotic organism and the genetic versatility of yeast have prompted researchers to set up target-specific HTS assays with engineered yeast cells. Functional assays for each of the four major classes of classical drug targets (ion channels, nuclear receptors, GPCRs and enzymes) have been established, thus underscoring the applicability of yeast as screening tool [13].

For example, the activity of the mammalian IRK1 protein, an inwardly rectifying K<sup>+</sup> channel, has been reconstituted in yeast. Expression of active IRK1 yields a growth response, which is readily assayed in a HTS format, by complementing the growth phenotype of a yeast mutant defective in K<sup>+</sup> uptake [14] (Bristol-Myers Squibb, <http://www.bms.com/>). A yeast cell-based assay has also been successfully applied to identifying inhibitors of the M<sub>2</sub> ion channel-forming protein of the influenza virus [15] (Bristol-Myers Squibb). Viral M<sub>2</sub> polypeptides associate as disulfide-linked homotetramers to permit the flow of protons from the endosome into the virion, resulting in a pH decrease and thereby stimulating viral uncoating in the early stage of the infection cycle. The growth-impairment phenotype resulting from M<sub>2</sub> expression in yeast was exploited for developing a HTS assay that led to the identification of an inhibitor, which was shown to block viral replication in a plaque reduction assay with an IC<sub>50</sub> of 0.26 μM [15]. In a different approach, the so-called reverse two-hybrid system has been adapted to HTS format for selecting compounds that block association of the N-type calcium channel β3 subunit with the α1B subunit, which is essential for its function [16] (Wyeth-Ayerst Research, <http://www.wyeth.com/>). Specific inhibitors identified in this screening assay have been subsequently shown to block the whole channel activity in functional assays with mammalian cells.

A further example is provided by yeast cell-based screening assays with human G-protein-coupled receptors (GPCRs). GPCRs have been functionally expressed in yeast, in which they can substitute for the structurally similar Ste2p, the yeast mating pheromone receptor. Thus, ligand binding to the human GPCR can activate the yeast pheromone-signaling pathway and trigger induction of mating-specific genes as well as activation of selectable reporter genes bearing mating-specific promoter elements. In a particularly elegant study, Klein *et al.* [17] identified surrogate agonists of the human formyl peptide receptor like-1 (FPRL-1) receptor by autocrine growth selection, that is by coupling FPRL-1 signaling to an

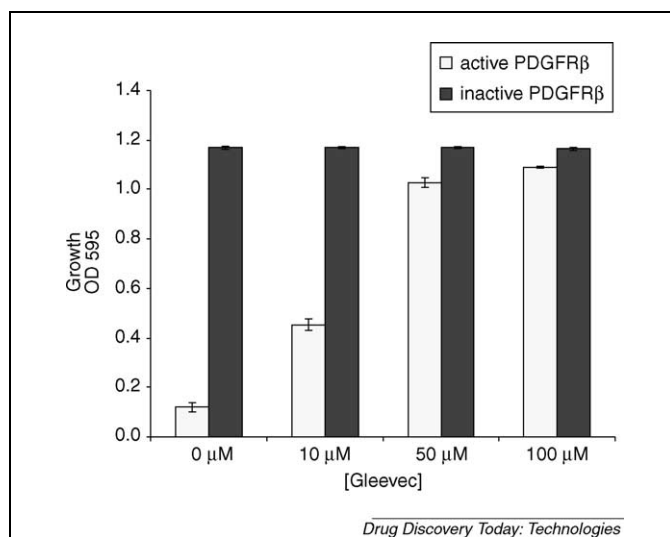
auxotrophic growth selection marker system (formerly Cadus Pharmaceuticals).

Yeast cell-based assays have also been developed and applied to HTS for the identification of inhibitors of enzymes as drug targets. For example, Perkins *et al.* [18] have reported the application of a yeast growth selection system for the identification of novel inhibitors of human poly(ADP-ribose) polymerase (PARP) (Iconix Pharmaceuticals, <http://www.iconixpharm.com/>). PARP activity in human cells is induced by double-strand DNA breaks. Thus, inhibition of PARP is believed to improve the efficacy of DNA-damaging agents as anticancer drugs in chemotherapy. Heterologous expression of PARP in yeast causes a growth reduction phenotype. A screen for compounds that can restore growth in PARP-expressing yeast cells identified several novel inhibitors that are specific for PARP1.

### Yeast assay for the identification of inhibitors of receptor tyrosine kinases

Aberrant signaling by receptor tyrosine kinases (RTKs) is associated with various diseases, including cancer, psoriasis, atherosclerosis and diabetes [19]. The validity of RTKs as therapeutic targets is illustrated by the success of the small molecule tyrosine kinase inhibitor Gleevec, which targets bcr-Abl in chronic myelogenous leukemia, c-kit in gastrointestinal stromal tumors and platelet-derived growth factor receptor  $\beta$  (PDGFR $\beta$ ) in chronic myelomonocytic leukemia.

Although biochemical assays have been successfully used to identify potent and specific tyrosine kinase inhibitors, they also have important limitations: First, most inhibitors identified by such screens primarily inhibit the active form of a kinase. As it has been shown for Gleevec, the adoption of an inactive conformation of the kinases bcr-Abl and PDGFR $\beta$  is essential for binding [20,21]. As opposed to biochemical assays, within cells a dynamic equilibrium between active and inactive conformations is maintained by the action of protein tyrosine phosphatases, thereby allowing identification of inhibitors that can function either by binding to the active conformation or by blocking the inactive state. Second, in the physiological environment of cells, there might be conformational characteristics of native RTKs that are not readily recapitulated in the *in vitro* assay. This is illustrated by the identification of two compounds that inhibited the insulin-like growth factor 1 receptor (IGF-1R) and the insulin receptor (INSR) with similar potencies in biochemical assays, whereas, in cell-based assays these compounds were highly selective for IGF-1R [22,23]. A plausible explanation for such discrepancy is that there are conformational differences between the native forms of these two RTKs that are not reproduced in the biochemical assay. These observations, taken together, indicate the need for the development of cell-based assays for the identification of RTK inhibitors that can be adapted to HTS.



**Figure 1.** PDGFR $\beta$ -expressing yeast cells are sensitive to Gleevec. Yeast cells lacking the three major ATP-dependent ABC transporters Pdr5p, Sng2p and Yor1p were exposed to a dose-dependent concentration series of Gleevec. The level of growth restoration was determined by OD<sub>595</sub> light scattering measurement. Addition of 100  $\mu$ M Gleevec completely restored growth of PDGFR $\beta$  expressing yeast cells.

Researchers in our laboratories have developed a yeast cell-based growth selection system by taking advantage of the fact that expression of activated human RTKs such as PDGFR $\beta$  inhibits growth of yeast cells in a kinase-dependent manner [24] (ESBATEch AG, <http://www.esbatech.com/>). This phenotype provides the basis for developing a positive growth selection system that selects for inhibition of kinase activity and against general cytotoxicity of compounds. Moreover, specificity of inhibitors can be rapidly addressed in yeast with a broad panel of human RTKs. In a proof-of-principle experiment, it was shown that Gleevec restored growth of yeast cells expressing PDGFR $\beta$  in a dose-dependent manner (Fig. 1). Importantly, the RTK specificity profile of Gleevec correlated well with published data (Fig. 2). In a further study in collaboration with Novartis, the IGF-1R inhibitors mentioned above were analyzed in yeast against a panel of 14 RTKs. Strikingly, the IGF-1R inhibitor that lacked selectivity *in vitro*, but was specific in mammalian cells, showed the same specificity in yeast cells (unpublished data). These results demonstrate that appropriately engineered yeast can serve as screening tool for identifying RTK inhibitors by combining advantages of *in vitro* (target-specific read-out) with those of cell-based assays (physiological environment).

### Yeast assay for the identification of cell-active inhibitors of $\beta$ -secretase

Aggregation of the amyloid- $\beta$  (A $\beta$ ) peptide in the brain has been implicated as the primary cause of Alzheimer's disease (for a review, see [25]). A $\beta$  peptide results from sequential

Human RTK	Growth stimulation by Gleevec
PDGFR $\beta$	+++
PDGFR $\alpha$	+++
MET	-
RON	-
IGF-1R	-
INSR	-
FGFR-1	-
TRKA	-
EPHB2	-
EPHB4	-
AXL	-
TEK	-
RET	-

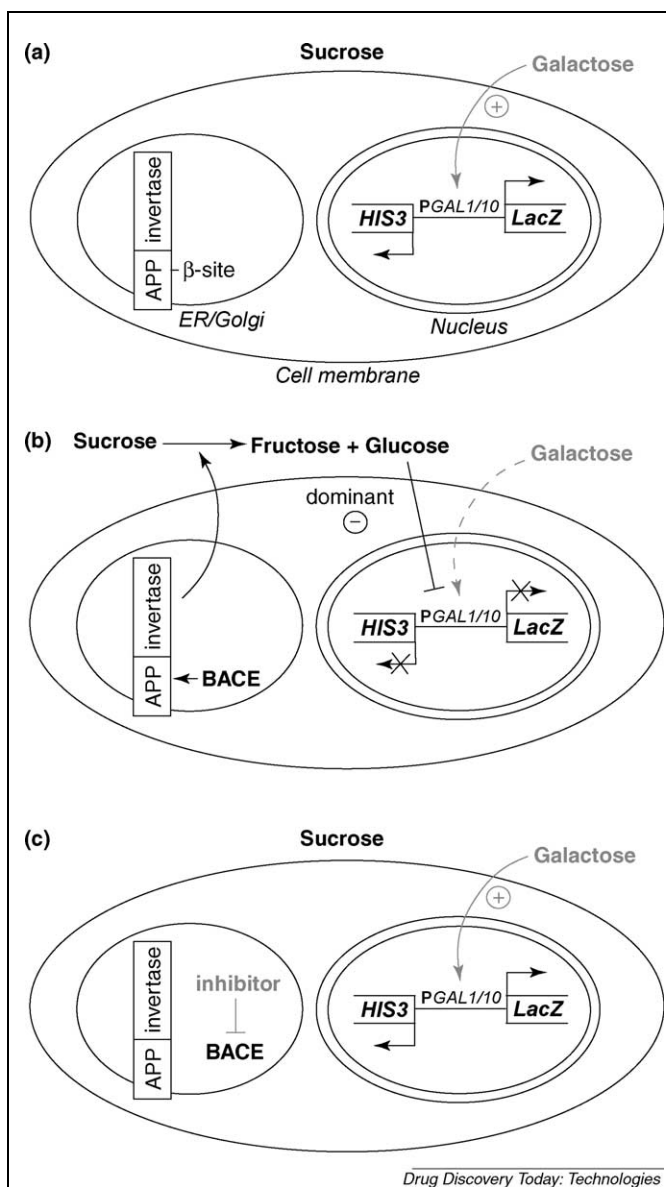
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**Figure 2.** Specificity profile of Gleevec in yeast cells expressing different human RTKs.

cleavage of the amyloid precursor protein (APP) by the  $\beta$ -secretase (BACE) and the  $\gamma$ -secretase. Because BACE knockout mice have significantly reduced A $\beta$  levels in the brain, but develop normally and show no apparent phenotype, BACE is considered an ideal therapeutic target for treating AD.

We have established a cellular growth selection system for functional expression of human BACE in yeast, in which processing of human APP by this membrane-bound protease in the ER–Golgi lumen is readily detected [26,27]. These yeast cells have been engineered so as to grow under selective conditions only upon reduced or inhibited BACE activity, thus providing a positive growth selection assay for BACE inhibitors (Fig. 3). Thus, these cells are currently used in HTS assays to select for compounds that inhibit this transmembrane secretase acting on its natural substrate in a membrane-bound protein configuration, a condition that would be very difficult to achieve in a biochemical assay.

In a recent proof-of-concept series of experiments performed in collaboration with Novartis, validated peptidomimetic BACE inhibitors, which reduced A $\beta$  secretion in a mammalian cellular assay with an IC<sub>50</sub> of 7  $\mu$ M, caused significant growth stimulation of the engineered yeast cells at the same compound concentration [28] (ESBATEch AG, <http://www.esbatech.com/>). Importantly, the same compounds did not stimulate growth of a yeast strain expressing the  $\alpha$ -secretase Yps1p instead of BACE, thus demonstrating the specificity of the system. By contrast, the nonspecific compound MG132, which has been shown to reduce A $\beta$  secretion in mammalian cells through a BACE-independent mechanism [29], stimulated growth of yeast cells expressing either BACE or Yps1p [28]. These results emphasize the reliability of the yeast assay and the importance of having readily at hand controls like Yps1p that allow rapid distinction between compounds that impair BACE function directly and false-positive compounds that interfere with the system in a nonspecific manner.



**Figure 3.** Yeast cell-based assay to select for BACE inhibitors. Each drawing depicts a schematic yeast cell that is grown in medium containing sucrose and galactose. Two cell organelles are indicated: The nucleus containing the reporter construct with the *HIS3* and *lacZ* genes under the control of the bi-directional *GAL1-10* promoter, and the ER/Golgi compartments harboring the membrane-anchored invertase–APP fusion protein. (a) State of the system in the absence of a secretase: Invertase is retained in the ER/Golgi compartments, sucrose remains inert to the system, and galactose induces transcriptional activation of the reporter genes. (b) BACE cleaves invertase–APP at the  $\beta$ -site of APP; the invertase enzyme is liberated and secreted to the periplasmic space of the cell, where it hydrolyses sucrose to generate fructose and glucose. The freshly generated glucose dominantly represses transcription of the reporter genes. (c) Addition of a BACE inhibitor restores induction of reporter gene expression by galactose, thus allowing cell proliferation under selective conditions.

## Conclusion

To enable the rapid analysis of large numbers of compounds for their effects on the function of an increasing number of potential therapeutic targets, fast and robust HTS systems are

**Table 1. Comparison summary**

Technology	Yeast cellular HTS	Mammalian cellular HTS	<i>In vitro</i> HTS	<i>In silico</i> HTS
<b>Pros</b>	<ul style="list-style-type: none"> <li>Genetic malleability and established wealth of genetic tools for analysis of biological functions</li> <li>Target protein is presented in its native conformation in a physiological environment</li> <li>Selection for membrane permeability and against cytotoxicity</li> <li>Clean read-out in a heterologous, yet eukaryotic environment</li> <li>Self-renewal system</li> <li>Simple handling</li> <li>Fast discrimination of real hits from false positives</li> </ul>	<ul style="list-style-type: none"> <li>Target protein is presented in its native conformation in a physiological environment</li> <li>Selection for membrane permeability and against cytotoxicity</li> <li>Eukaryotic environment</li> <li>Self-renewal system</li> </ul>	<ul style="list-style-type: none"> <li>A well-optimized assay has less data scatter than a cellular assay</li> <li>Isolated target simplifies follow-up (SAR analysis) after screen</li> <li>High compound concentration aids in the identification of novel chemical classes</li> </ul>	<ul style="list-style-type: none"> <li>Fast</li> <li>Reduction of number of compounds to be measured in secondary assays</li> <li>Requested properties of the compounds entering the HTS can be freely determined</li> </ul>
<b>Cons</b>	<ul style="list-style-type: none"> <li>Reduced sensitivity to defined compound classes owing to efficient drug efflux pumps</li> <li>No established protocols and limited knowledge about yeast screens in the scientific community</li> </ul>	<ul style="list-style-type: none"> <li>Function of the target protein is influenced by redundant cellular processes</li> <li>Difficulty to discriminate real hits from false positives</li> <li>Difficult and time-consuming genetic manipulations</li> </ul>	<ul style="list-style-type: none"> <li>Target protein has to be purified and biochemically characterized</li> <li>Larger series of chemical starting points because hits obtained are not initially required to possess cell permeability and reduced cytotoxicity</li> <li>No self-renewal system</li> <li>Suitable substrate must be identified and synthesized</li> </ul>	<ul style="list-style-type: none"> <li>Crystal structure has to be determined (or already publicly available)</li> <li>Rigid model of the receptor. Current docking programs are unable to model loop movements or induced-fit effects</li> <li>No experimental data, only predicted</li> </ul>
<b>Costs</b>	<ul style="list-style-type: none"> <li>Inexpensive culture conditions</li> </ul>	<ul style="list-style-type: none"> <li>Expensive culture conditions</li> </ul>	<ul style="list-style-type: none"> <li>Expensive: protein purification, synthesis of substrate</li> </ul>	<ul style="list-style-type: none"> <li>Inexpensive</li> </ul>

required. In the past few years, however, it has become clear that one condition for successful drug development is the selection of compounds, or 'scaffolds', endowed with so-called drug-like properties already during the early stages of the discovery process. In this sense, cell-based assays that are applicable to HTS have clear advantages over biochemical assays because they immediately select for at least some of such properties, namely membrane permeation and stability in the intracellular environment (see Table 1). The easy genetic manipulation of yeast and the high degree of conservation of basic molecular mechanisms with human cells have made this eukaryotic microorganism a valuable tool for target-specific drug screening. As shown by several research groups in academic institutions as well as in pharmaceutical and biotech companies, yeast cell-based assays developed for HTS have been successfully applied to identify inhibitors of enzymes, antagonists of ion channels, and protein interaction blockers. Yeast cell-based assays offer the opportunity to screen for inhibitors (or activators) of human targets in a heterologous eukaryotic environment lacking redundant cellular processes that often interfere with a clean read-out of target-specific effects. We have performed HTS against RTKs as well as the secretase BACE and shown that small molecules with activities in the low  $\mu\text{M}$  range in secondary human cellular assays could be identified from a 15,000 compound chemical library.

The technical advantages of yeast cell-based HTS assays described here, taken together with the importance of drug-like properties of compounds selected by cellular systems in general, are expected to expand the application of yeast assays even further in the field of drug discovery. It can be argued that the full potential of yeast cell-based assays in HTS will become more apparent in correlation with the increasing number of potential therapeutic targets that can enter the drug discovery and development processes.

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