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Yeast as a model system to screen purine derivatives against human CDK1 and CDK2 kinases

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ABSTRACT

Cyclin-dependent kinases (Cdk) play crucial roles in cell cycle progression. Aberrant activation of Cdk1 has been observed in a number of primary tumors and Cdk2 is deregulated in various malignancies. The therapeutic value of targeting Cdk1 and Cdk2 has been explored in a number of experimental systems. In the present study, taking advantage of the fact that deletion of the yeast *CDC28* gene is functionally complemented by human *CDK1* or *CDK2*, we set up an *in vivo* screen system to evaluate the inhibitory potency of purine derivatives against these two human Cdks. We constructed three isogenic strains highly sensitive to small molecules and harboring genes *CDK1*, *CDK2* or *CDC28*, under the control of the *CDC28* promoter. In a proof of principle assay, we determined the inhibitory effect of 82 purine derivatives on the growth rate of these strains. Thirty-three of them were revealed to be able to inhibit the Cdk1- or Cdk2-harboring strains but not the Cdc28-harboring strain, suggesting a specific inhibitory effect on human Cdks. Our data demonstrate that the yeast-based assay is an efficient system to identify potential specific inhibitors that should be preferentially selected for further investigation in cultured human cell lines.

1. Introduction

Protein kinases control many cellular processes such as signal transduction, cell cycle, transcription, metabolism, cytoskeletal dynamics, differentiation and apoptosis. The human kinome contains 518 kinases (Manning et al., 2002). Among these kinases, cyclin-dependent kinases (CDK) and CDK-related kinases form a family of 21 highly homologous members (Malumbres et al., 2009). CDKs play crucial roles in cell cycle progression (CDK1, 2, 3, 4, 6, 7), in neuronal functions (CDK5) and in transcription (CDK7, 8, 9) (Knockaert et al., 2002a; Meijer, 2000). Mutations and dysregulation of protein kinases are causes of many human diseases and these enzymes have become targets for low-molecular weight therapeutic agents (Blume-Jensen and Hunter, 2001; Cohen, 2002; Fabbro et al., 2002). Particularly, CDK inhibitors appear to be promising therapeutic agents for the treatment of cancer, neurodegenerative diseases and viral infections (Knockaert et al., 2002a; Meijer, 2000; Nabel, 2002; Schang, 2003).

Multiple methods are available for profiling the activity of kinase inhibitors *in vitro* and in cells (Smyth and Collins, 2009). *In vitro* approaches give insight into the affinities of small molecules for their kinase targets. One method consists in the determination of their *in vitro* effects on a panel of purified kinases (Bain et al., 2003). Affinity chromatography on immobilized inhibitors represents another manner to identify drug targets (Knockaert et al., 2002b). cDNA phage display technology (Sche et al., 1999), yeast three-hybrid system (Becker et al., 2004), *in vitro* ATP site-dependent competition binding assays (Fabian et al., 2005) or chemical genomic method (Kung et al., 2005) are other ways to test the selectivity of protein kinase inhibitors. Cell-based screens have important advantages over *in vitro* assays because the activity of the target protein, as well as the potential effects of compounds, could

Abbreviations: CDK, cyclin-dependent kinase; cDNA, complementary DNA; DMSO, dimethylsulfoxide; ABC transporter, ATP-binding cassette transporters; GI_{50}, 50% growth inhibition; IC_{50}, half maximal inhibitory concentration.

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be analyzed in a cellular context that simulates better the normal physiological state than *in vitro* assays (Barberis et al., 2005). Cellular screens should preferentially be performed with human cells. However, genetic manipulation of mammalian cells is generally tricky and time consuming.

Yeast cell-based assays represent an attractive model system for drug screenings. In yeast cells the function of human proteins can often be reconstituted and aspects of some human physiological processes can be recreated because of the high degree of conservation of basic molecular and cellular mechanisms between yeast and human cells. Furthermore, genetic manipulation can be easily performed in yeast. Indeed, yeast cell-based assays to screen for inhibitors of human enzymes are widely utilized (Simon and Bedalov, 2004; Barberis et al., 2005). For instance, yeast-based specific phenotypic screens can be designed for proteins that do not have yeast homologues. Such heterologous proteins (*e.g.* human kinases) expressed in yeast cells may induce growth defects. Drugs that restore yeast growth by inhibiting the activity of the heterologously expressed protein can be positively selected. For foreign protein complementing a yeast orthologue, the selection screen is negative and the inhibition of the target leads to changes in the rate of cell proliferation. In this case, an appropriate demonstration that the observed phenotype is not due to a non-specific toxic effect should be considered. The next step consists to validate the inhibitors identified from yeast-based screens in mammalian cells that express the target genes.

Cdc28 cyclin-dependent kinase of *Saccharomyces cerevisiae* is the major regulator of the cell cycle (Mendenhall and Hodge, 1998). Cdc28 is homologous to human CDK1, 2 and 3 (Liu and Kipreos, 2000). Cdc28 mutants are functionally complemented by human CDK1 (Wittenberg and Reed, 1989) and CDK2 (Elledge and Spottswood, 1991; Ninomiya-Tsuji et al., 1991; Tsai et al., 1991). In the present study, we have constructed three strictly isogenic yeast strains which were able to proliferate thanks to the presence of Cdc28, human Cdk1 (hsCdk1) or human Cdk2 (hsCdk2) kinases. We have set up an *in vivo* screen to evaluate the inhibitory potency and specificity of tri-substituted purine derivatives using these three strains. Tri-substituted purines are acting by competing with the binding of ATP in the ATP-binding pockets of the targeted CDKs/kinases (Fischer et al., 2003). The purine derivatives tested were synthesized previously and for most of the compounds, their IC_{50} values on purified Cdk1/cyclin B from starfish oocytes were already described (Ducrot et al., 2000; Legraverend et al., 1998, 2000, 2001).

2. Materials and methods

2.1. Strain constructions

The constructions of plasmids and strains used in this study are described in Supplementary data.

2.2. Growth tests

Growth tests were done in YPD (1% Yeast Extract, 1% Bacto Peptone, 2% glucose). The concentration of stock solutions of purine derivatives was 10 mg/ml (about 25 mM) in 100% DMSO. Stock solutions were diluted 10 and 50 times in DMSO before treating strains GF4351, GF4356 and GF4359. Yeast strains were grown in YPD medium to early logarithmic phase (OD at 595 nm: 0.130–0.150, Beckman DU 640 B spectrophotometer). Culture aliquots (98 μl) were added immediately to 96-wells Microtest tissue culture plates with flat bottom and low evaporation lid (Falcon # 353072). Then, 2 μl of DMSO, with or without (control) purine analog dilutions, were added in the YPD cultures (final volume: 100 μl). All the screenings were done in duplicate. The plates were agitated on a shaker (Heidolph Titramax 1000) at 28 °C. The absorbance of each culture was measured every 2 h for the first 12 h with a Bio-Rad Model 550 Microplate Reader (wavelength: 595 nm). Doubling times were determined and the percentages of inhibition were calculated using the following equation: $\%\text{inhibition} = (1 - D_1/D_2) \times 100$, where D_1 is doubling time with no compound and D_2 is doubling time with test compound. The percentages of inhibition were plotted onto log Probit graph paper (%inhibition (Probit scale) *versus* logarithmic concentration of test compound) to estimate the compound concentrations that yield a growth rate which is 50% of the maximum rate (GI_{50}) (Litchfield and Wilcoxon, 1949).

3. Results

3.1. Yeast strain constructions

We first constructed yeast strains permeable to small molecules. Enhanced sensitivity of yeast cells to drugs can be achieved by deleting pleiotropic drug ABC transporters that extrude drugs out of the cells, or genes that regulate their expression (Bauer et al., 2003; Kolaczkowska and Goffeau, 1999; Rogers et al., 2001). ABC transporters Pdr5 and Snq2 are involved in multidrug resistance. The deletions of their encoding genes increase the toxicity of a large spectrum of compounds (Kolaczkowski et al., 1998). *PDR5* and *SNQ2* genes were therefore deleted with the KanMX disruption cassette (Guldener et al., 1996), resulting in strain GF3997 (*pdr5*Δ *snq2*Δ). On the other hand, genes *PDR1*, *PDR3* and *YAP1* code for regulatory factors of two networks of genes involved in multidrug resistance (Kolaczkowska and Goffeau, 1999; Rogers et al., 2001), these three genes were also deleted using the disruption cassette *hisG-URA3-hisG* which allows the repeated use of *URA3* selection (Alani et al., 1987), resulting in strain GF4225 (*pdr1*Δ *pdr3*Δ *yap1*Δ). The gain of sensitivity to cycloheximide, ketoconazole or Triton X-100 by strains GF3997 (*pdr5*Δ *snq2*Δ) and GF4225 (*pdr1*Δ *pdr3*Δ *yap1*Δ) compared to the starting strain GF2275 was assessed with an agar diffusion assay (Supplementary Fig. S1) as previously described (Kolaczkowski et al., 1998). Finally, in order to obtain strains highly hypersensitive to many drugs, a strain carrying *pdr5*Δ *snq2*Δ was crossed with a strain carrying *pdr1*Δ *pdr3*Δ *yap1*Δ, followed by sporulation and tetrad dissection. Two isogenic strains, GF4253 and GF4254 with genes *PDR5*, *SNQ2*, *PDR1*, *PDR3* and *YAP1* all deleted, were retained. Strains GF4253 and GF4254 were crossed to obtain a diploid strain designated GF4271. Then, one of the two *CDC28* genes of GF4271 was removed using the disruption cassette *hisG-URA3-hisG* (Alani et al., 1987), resulting in strain GF4301.

Three integrative plasmids, p1305, p1306 and p1309, harboring genes *CDK1*, *CDK2* and *CDC28*, respectively were constructed (Supplementary Fig. S2). The *CDK1* and *CDK2* genes are under the control of *CDC28* promoter and *CYC1* terminator, while *CDC28* is under the control of its own promoter and terminator. These plasmids bear the *HIS3*MX6 cassette (Longtine et al., 1998) as a selective marker and the *LEU2* gene. After digestion with appropriate restriction enzymes cleaving inside the *LEU2* coding sequence, the plasmid DNAs were inserted by homologous recombination at the *LEU2* locus of the diploid strain GF4301. After sporulation and tetrad analysis, segregants GF4351, GF4356 and GF4359 (Supplementary Table S1) were retained. These three strains are entirely isogenic, except that the *cdc28* deletion is complemented by genes *CDK1*, *CDK2* or *CDC28*, respectively. Importantly, *CDK1*, *CDK2* or *CDC28* genes are placed in the same chromosomal context and expressed under the control of the same promoter. The doubling times of these strains were the following: GF4359 (Cdc28), 2 h 15; GF4351 (hsCdk1), 2 h 45; and GF4356 (hsCdk2), 5 h 30 (Supplementary