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# Gefitinib induces mitochondrial-dependent apoptosis in *Saccharomyces cerevisiae*

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**Abstract.** Gefitinib, a selective inhibitor of the epidermal growth factor receptor (EGFR) tyrosine kinase, has been clinically demonstrated to be effective in certain cancer cell types. In the present study, using the yeast *Saccharomyces cerevisiae* as a model, gefitinib-induced apoptotic cell death was demonstrated. Gefitinib inhibited yeast cell proliferation and ultimately led to cell death in a time- and dose-dependent manner. Furthermore, when cells were exposed to 15 $\mu$M gefitinib, typical apoptotic markers, including phosphatidylserine exposure, DNA fragmentation, reactive oxygen species production and decrease in mitochondrial membrane potential, were observed. The *Δcyc3* strain deleted in cyt c heme lyase and the rho$^0$ mutant strain lacking mtDNA-delayed cell death, provided further evidence that the yeast cell death process involved the mitochondria. Thus, these findings suggest that gefitinib induces apoptosis in yeast cells through a mitochondrial-dependent pathway.

## Introduction

Apoptosis is a highly coordinated cellular suicide program that is crucial for the maintenance of health and tissue function, and alterations in this elaborate cellular homeostasis mechanism lead to cancer or neurodegenerative disorders. Scientific research has pointed out that the mechanism by which most of the anti-tumour drugs kill tumour cells involves the induction of cell death by apoptosis (1). In fact, the increase in knowledge on apoptosis itself, particularly its regulation in cancer cells, has identified various possible targets for therapeutic intervention in cancer (2). Consequently, great interest has emerged in developing new strategies; that is, the elimination of cancer cells by the preferential induction of apoptosis in these cells while sparing normal cells, thereby offering an exciting multitude of molecular targets and therapeutic options for the future (3).

Recently, yeast was established as a model for studying the mechanisms of apoptotic regulation (4). In the yeast *Saccharomyces cerevisiae*, cell death with typical markers of apoptosis was detected, such as phosphatidylserine externalization, DNA fragmentation and chromatin condensation. Several crucial regulators of apoptosis are conserved between metazoans and yeast; for example, Yca1p plays a central role in yeast apoptosis, characterizing it as an orthologue of caspases and as a mammalian classical apoptotic regulator (5). Many intriguing aspects of apoptosis can be approached more effectively in the simpler eukaryotic cell *S. cerevisiae* than in human cells. *S. cerevisiae* is a facultative anaerobic yeast and tolerates even the complete loss of its mitochondrial DNA (rho$^0$ strains), which is especially helpful for research on apoptosis, as mitochondria play a central and complex role in apoptosis (6). The mechanisms of numerous anti-cancer drugs, inducing apoptosis in yeast, have been investigated. Arsenic has been shown to induce DNA fragmentation, phosphatidylserine exposure, mitochondrial membrane permeabilization and reactive oxygen species (ROS) accumulation in *S. cerevisiae* cells, as well as decrease cell death in a Yca1p-dependent manner (7). Bleomycin-induced apoptotic cell death in yeast is mainly characterized by the appearance of a sub-G0/G1 population, the generation of DNA double-strand breaks and, at high bleomycin concentrations, the induction of a mitochondrial-independent cell death process (8). Edelfosine has been reported to promote apoptosis in *S. cerevisiae* cells characterized by a TUNEL-positive phenotype and mitochondrial-dependent ROS generation, presenting similarities with edelfosine-induced apoptosis in human tumour cells (9).

Gefitinib (Iressa®, ZD1839, AstraZeneca) is a small-molecule anilinoquinazoline compound, originally described as an inhibitor of the tyrosine kinase of the epidermal growth factor receptor (EGFR), which is overexpressed in most human solid tumours. Gefitinib has broad anti-tumour effects against various types of solid tumours, including non-small cell lung cancer, breast, ovarian cancer and head cancers (10). Although the induction of apoptosis has been considered as a major mechanism in the anti-cancer effects of gefitinib, most of these effects are EGFR-dependent. Gefitinib has been reported to inhibit the EGF-triggered pathway and the

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constitutive HER3-mediated Akt activation in chemoresistant cells (ovarian cancer), and to block the EGF-induced phosphorylation of ErbB-1 and mitogen-activated protein kinase (MAPK) to decrease cell proliferation in different pancreatic cancer cell lines (11,12). However, gefitinib has been reported to induce cell cycle arrest at the G1/S checkpoint in hepatocellular carcinoma, and the accumulation of cells in the G0/G1 phase to delay human gallbladder adenocarcinoma cell growth (13,14). Gefitinib has also been used in combination with other chemotherapeutic agents to enhance the anti-cancer effect. Rosano *et al* found that the combination of gefitinib and ZD4054 in the treatment of ovarian carcinoma not only maximally inhibited cell proliferation, but also inhibited multiple downstream signaling pathways, including MAPK, phosphoinositide 3-kinase-dependent (PI-3K), integrin-linked-kinase and src-mediated EGFR transactivation, finally arresting the metastatic spread of cancer (15). Therefore, the mechanisms of gefitinib-induced apoptosis in cancer cells are mainly focused on the blocks of signal transduction pathways implicated in the proliferation and survival of cancer cells, as well as other host-dependent processes that promote cancer growth. Lindhagen *et al* proposed that gefitinib has significant cytotoxic activity in acute myeloblastic leukaemia and that it induces apoptosis through non-EGFR-dependent pathways (16). However, there have not been many investigations on the molecular mechanisms related to the cytotoxic activity of gefitinib in normal cells. In order to reduce the cytotoxicity of the drug, the molecular mechanism for gefitinib-induced apoptosis remains to be fully elucidated.

In the present study, using yeast as a model system, we provided evidence that gefitinib potently inhibits cell growth and induces apoptosis in *S. cerevisiae* via the mitochondrial-dependent pathway.

## Materials and methods

*Chemicals, yeast strains and growth conditions*. Gefitinib (Iressa, ZD1839) was kindly provided by AstraZeneca Ltd. (Shanghai, China) and was dissolved in dimethyl sulfoxide (DMSO). Experiments were carried out in the *S. cerevisiae* strain, BY4741 (MATa, ura3Δ0, leu2Δ0, met15Δ0, his 3Δ1), and respective null mutant strains, including *Δcyc3* and $rho^0$ obtained from Euroscarf. All strains were pre-grown for 24 h in glass tubes containing SCD medium consisting of 0.17% yeast nitrogen base, 0.5% $(NH_4)_2SO_4$, 80 mg/l histidine, 200 mg/l leucine and 30 mg/l of the rest of the amino acids, 30 mg/l adenine and 320 mg/l uracil with 2% glucose. The cultures were then inoculated to 10 ml of YPD medium (1% yeast extract, 2% peptone and 2% glucose at 30˚C) in 50 ml flasks incubated on a mechanical shaker (200 rpm) at 30˚C.

*Cell growth and viability*. To determine the effect of gefitinib on cell growth, cells cultured to the mid-exponential phase were adjusted to $1.0x10^7$ cells/ml, and inoculated in 24-well plates with YPD medium containing various concentrations of gefitinib (0-30 $\mu$M) at a final volume of 1 ml per well. Cell numbers were calculated every 12 h for 96 h using a haemocytometer chamber. Cell viability was determind by colony forming unit (CFU) counts, the yeast culture was adjusted to an identical OD600 nm, and additional 10-fold dilutions were made in the YPD medium. Then, ~200 cells were calculated and spread onto YPD plates containing various concentrations of gefitinib. The number of surviving colonies was determined after 48 h of incubation at 30˚C.

*Terminal deoxynucleotidyl transferase-mediated dUTP nick-end-labeling (TUNEL) assay*. DNA strand breaks were demonstrated by TUNEL with the *In Situ* Cell Death Detection kit (Roche Diagnostics, Germany) as described by Du *et al* with minor modifications (7). Briefly, yeast cells were fixed with 3.7% (v/v) formaldehyde for 1 h at room temperature, washed three times with phosphate buffered saline (PBS) and then digested with snailase at 37˚C for 1 h. Following the incubation with permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate) for 2 min on ice, the cells were washed and resuspended in TUNEL reaction mixture for 1 h at 37˚C in the dark. Finally, the cells were washed three times with PBS and analyzed under a fluorescence microscope (Olympus, BX50).

*Annexin-V fluorescein-isothiocyanate (FITC) staining*. To examine the exposure of phosphatidylserine on the outer leaflet of the plasma membrane, FITC-coupled annexin V (Annexin V Apoptosis kit; Clonetech Laboratories Inc.) was performed according to the manufacturer's instructions. In brief, yeast cells were washed in sorbitol buffer (1.2 M sorbitol, 0.5 mM $MgCl_2$, 35 mM potassium phosphate, pH 6.8), digested with 15 U/ml lyticase in sorbitol buffer for 1 h at 30˚C, harvested, washed in binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) containing 1.2 M sorbitol, harvested and re-suspended in binding buffer. Annexin-FITC (2 $\mu$l) and 1 ml of propidium iodide (PI; 500 mg/ml) were added to 40 $\mu$l of cell suspension and incubated for 30 min at room temperature. Cells were washed, re-suspended in binding buffer and then observed under a fluorescence microscope (Olympus, BX50).

*ROS production*. ROS production was detected with the fluorescent probe, DCFH-DA (Reactive Oxygen Species Assay kit; Beyotime), according to the manufacturer's instructions. Briefly, DCFH-DA at a final concentration of 10 $\mu$M/ml was added to 0.5 ml of cell suspension (~$1x10^7$ cells) in treated medium, followed by incubation at 37˚C for 20 min and then the cells were washed three times with PBS to remove the DCFH-DA which had not entered the cells. The production of free intracellular radicals was analyzed using a fluorescence activated cell sorter (FACScalibur; Becton-Dickinson) at a low flow rate with an excitation at 488 nm.

*Assessment of mitochondrial transmembrane potential (ΔΨ)*. The assessment of the changes of ΔΨ, was performed using rhodamine-123 (Rh123) staining (Cell Apoptosis Rhodamine 123 Detection kit; KeyGEN) according to the manufacturer's instructions. Cells (~$1x10^6$) were collected and re-suspended in 1 ml of HEPES buffer (20 mM, pH 7.4). Then, Rh123 at a final concentration of 5 $\mu$g/ml was added to the cell suspension and incubated at 37˚C for 15 min, centrifuged, washed and re-suspended in 1 ml of YPD medium for another incubation at 37˚C for 30 min. ΔΨ was expressed as the fluorescence intensity of Rh123, which was analyzed by flow cytometry

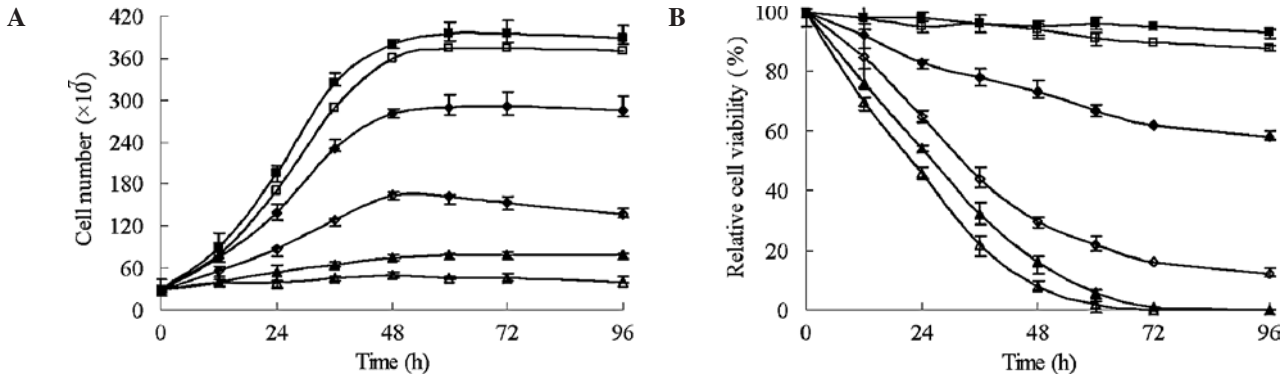


Figure 1. Effect of gefitinib on cell growth and viability. (A) The yeast *S. cerevisiae* BY4741 was cultured in YPA medium containing various concentrations of gefitinib at 30˚C and the growth was monitored by counting the cell number every 12 h for 96 h using a haemocytometer chamber. (B) The relative cell viability was evaluated by CFU counts (100% corresponds to the number of cells at time zero) in YPD plates. (■) 0 $\mu$M, (□) 5 $\mu$M, (♦) 10 $\mu$M, (◇) 15 $\mu$M, (▲) 25 $\mu$M and (△) 30 $\mu$M gefitinib. Values are the means ± SEM of a representative experiment of three independent experiments.

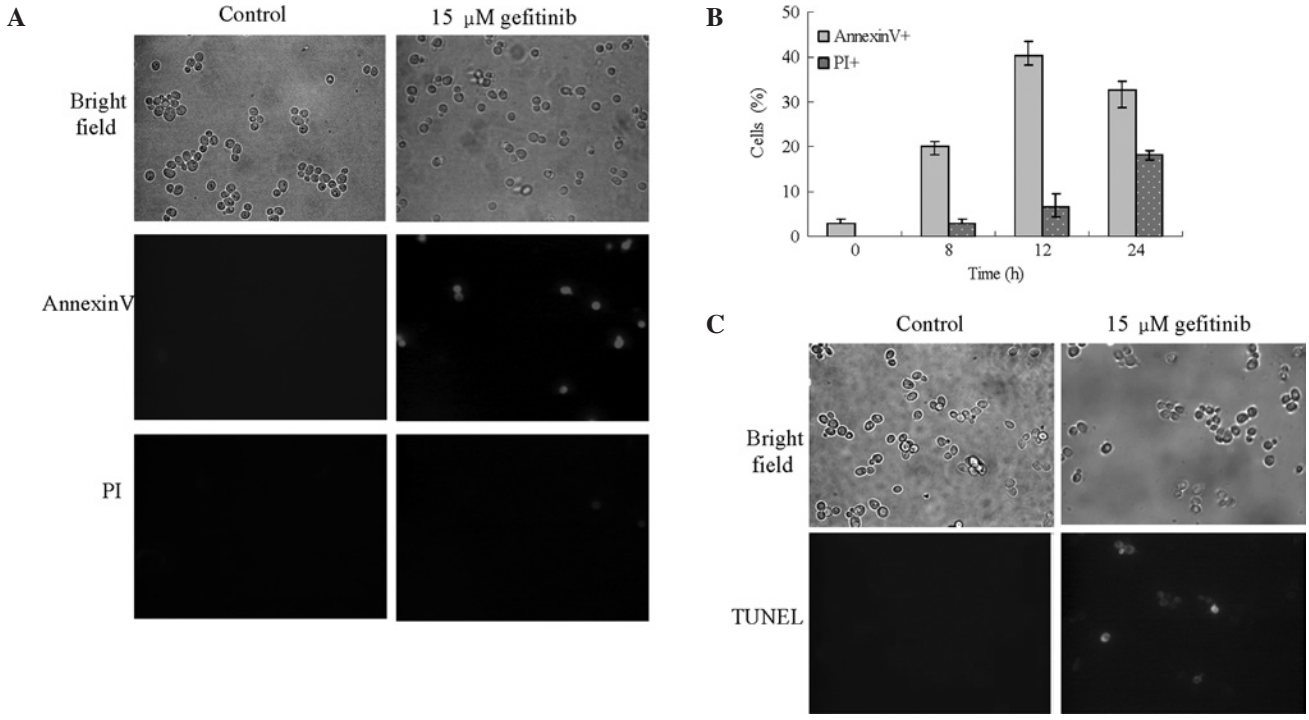


Figure 2. Gefitinib induction of yeast cell death showing apoptotic markers. (A) BY4741 cells were treated with 15 $\mu$M gefitinib for 12 h and stained with FITC-coupled annexin V together with PI to check the integrity of the plasma membrane. (B) Percentage of cells displaying phosphatidylserine extroversion (annexin $V^+$) and loss of membrane integrity ($PI^+$) as detected by FITC-annexin V assay and PI staining, respectively, after exposure to 15 $\mu$M of gefitinib for different times (8-24 h). To determine the percentage of positive cells, at least 300 cells were evaluated for each parameter. Experiments described in A, B and C were repeated three times with good consistency. Typical results are sited. (C) BY4741 cells were treated with 15 $\mu$M gefitinib for 16 h and stained with TUNEL.

with an excitation at 488 nm, or observed by a fluorescence microscope.

## Results

*Gefitinib inhibits cell growth and induces cell death of S. cerevisiae*. To examine the effect of gefitinib on the growth of yeast, the cells in the mid-exponential phase were incubated in YPD medium containing various concentrations of gefitinib (1-30 $\mu$M). The growth rates were monitored by counting the cell number every 12 h for 96 h. As shown in Fig. 1A, the growth of the strain in the presence of 15 or 30 $\mu$M gefitinib was increased during the first 48 h, but then decreased, and at 96 h, the cell numbers were 35.6 and 10.3%, respectively, of those observed in the absence of gefitinib. The results suggest that gefitinib inhibits cell growth in a dose-dependent manner. The cfu counting assay was carried out to detect cell death. The results showed that the relative cell viability exposed to 15 $\mu$M gefitinib was decreased to 12.3% within 96 h compared to that of the control, and that almost all cells died within 72 h when cultures contained 25 and 30 $\mu$M gefitinib (Fig. 1B), demonstrating that gefitinib induced budding yeast cell death in a dose- and time-dependent manner.

*Characteristic markers of apoptosis in cells treated with gefitinib*. To further investigate whether the gefitinib-induced

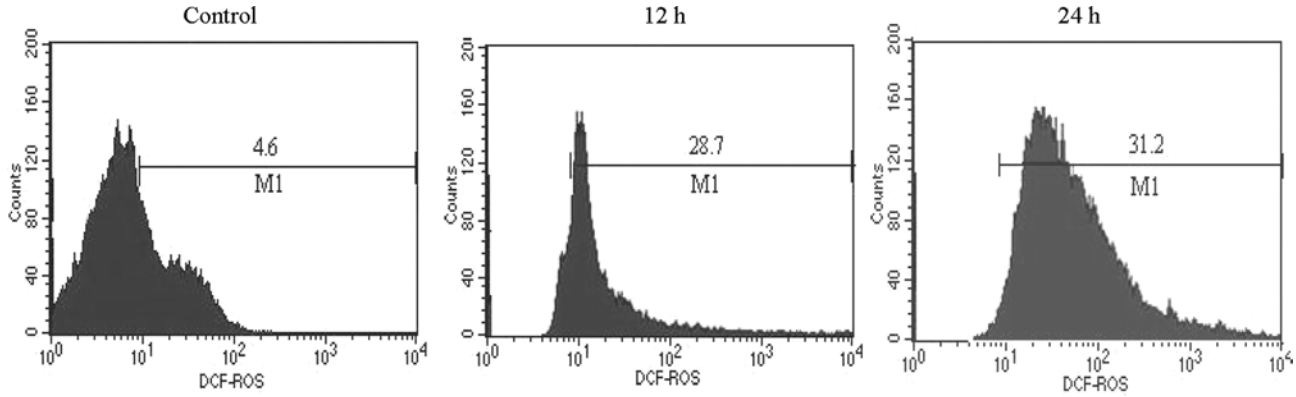


Figure 3. Effect of gefitinib on the generation of intracellular ROS in BY4742 cells. Cells were treated in the absence or presence of 15 $\mu$M gefitinib for 12 and 24 h, respectively, and analyzed by FACS using DCFH-DA dye. The experiment was repeated three times with good consistency. Typical results are sited.

cell death is apoptotic in nature, we examined cells for some apoptotic markers. Firstly, the exposure of phosphatidylserine on the plasma membrane is an early event in apoptosis and can be detected by staining with FITC-labeled annexin V, a protein with a strong affinity for phosphatidylserine. Treatment of *S. cerevisiae* with 15 $\mu$M gefitinib for 12 h resulted in 40.3% of the cells being annexin V-positive, whereas those of the control were negative, indicating the phosphatidylserine translocation to the outer membrane during gefitinib treatment (Fig. 2A).

Secondly, preservation of plasma membrane integrity is also one of the characteristic markers of apoptotic death, which is frequently assessed by PI staining. This dye penetrates the membranes of dead or dying cells, but it generally avoids viable cells. The gefitinib-inhibited *S. cerevisiae* growth was not accompanied by a significant loss of plasma membrane integrity. Indeed, after 8, 12 and 24 h of incubation with 15 $\mu$M gefitinib, only ~3, 6.5 and 18.2% of cells, respectively, lost their membrane integrity, while ~20.1, 40.3 and 32.6%, respectively, were annexin V-positive (Fig. 2A and B), suggesting that most cells were dying by apoptosis rather than by necrosis.

Thirdly, the TUNEL assay was used to determine the effect of gefitinib on DNA fragmentation in *S. cerevisiae* (Fig. 2C). The results showed that the 15 $\mu$M gefitinib-treated cells displayed nuclear green fluorescence, indicating the occurrence of DNA fragmentation. The percentage of the gefitinib-treated cells displaying TUNEL-positive staining increased over time reaching a maximum at 16 h (39.7%). However, TUNEL-positive cells were rare in the control cells.

*Gefitinib induced ROS production*. ROS production has been reported to be a key event in establishing yeast apoptosis (17). To investigate whether oxygen stress plays an important role in gefitinib-induced apoptosis, we determined the accumulation of ROS by flow cytometry using the dye, 2',7'-dichlorodihydrofluorescein-diacetate (DCFH-DA), which enters the live cell and is oxidized by ROS, emitting green fluorescence (18). Flow cytometric analysis showed that ~28.7 and 31.2% of yeast cells displayed significant ROS production after 12 and 16 h of treatment with 15 $\mu$M gefitinib, respectively, while most of the untreated samples did not show any fluorescence (Fig. 3). The results demonstrate that the ROS level was increases during gefitinib-induced *S. cerevisiae* apoptosis.

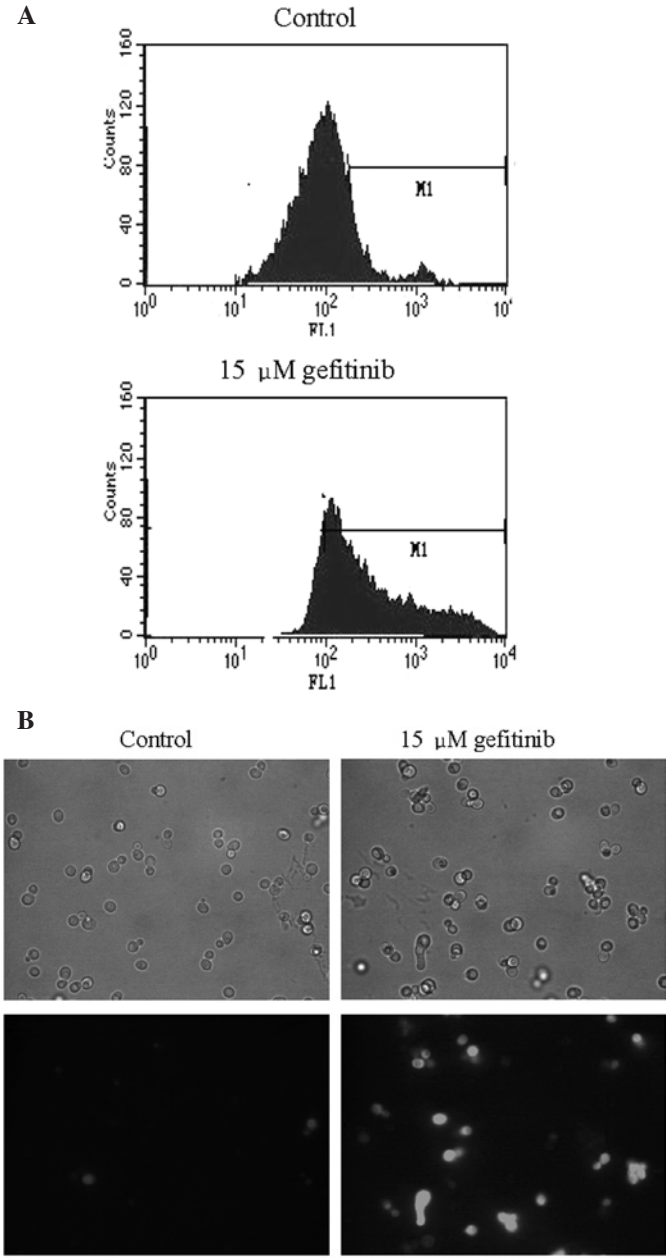


Figure 4. Effect of gefitinib on ΔΨ reduction in BY4742 cells. Cells were treated in the absence or presence of 15 $\mu$M gefitinib for 16 h. ΔΨ was measured using Rh123 by FACS analysis (A), and also observed under the fluoresce-detection optical system (B).

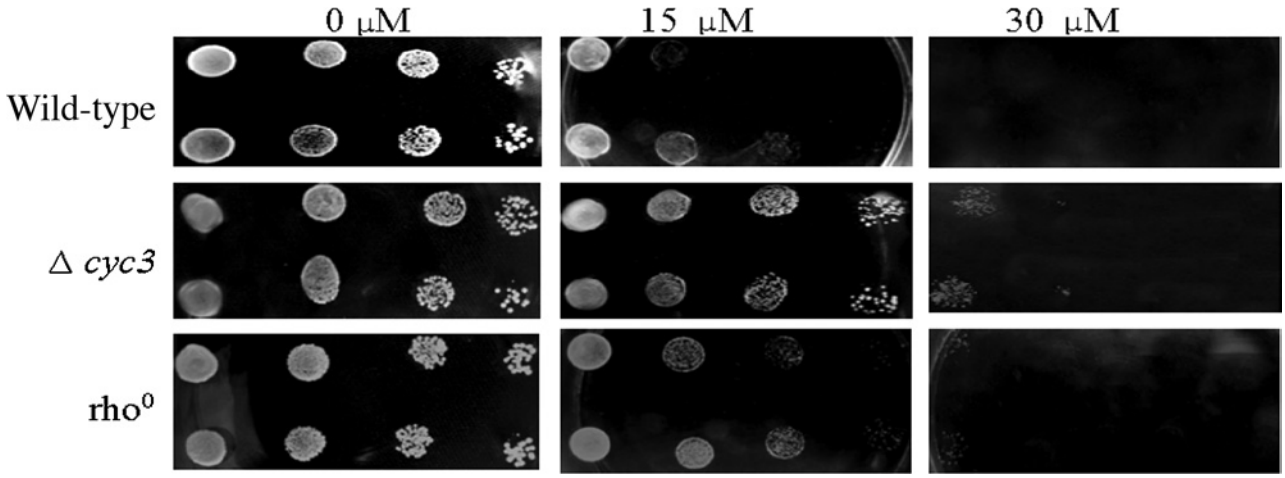


Figure 5. Viability of the *Δcyc3* and rho$^0$ strains compared to the wild-type in the absence or presence of gefitinib. Cells were treated with 0, 15 or 30 $\mu$M gefitinib for 48 h and known numbers of cells were spotted on a plate containing YPD medium after sequential dilution.

*Gefitinib decreases ΔΨ.* The mitochondria play an important role in yeast apoptosis (19). The loss of ΔΨ is considered to be an early event of apoptosis. To detect the levels of ΔΨ in cells exposed to gefitinib, cells were collected at different times and stained with Rh123. The results showed that many cells treated with 15 $\mu$M gefitinib for 12 h were green fluorescence-positive compared to the control cells (Fig. 4A). Similarly, FACS analysis also showed that the fluorescence intensity in the gefitinib-treated cells was higher than that in the control cells (Fig. 4B), confirming the ΔΨ disruption in gefitinib-induced cell death.

*Gefitinib-induced cell apoptosis is mitochondrial-dependent.* Further confirmation of the mitochondrial function in apoptosis induced by gefitinib was achieved by the study of two *S. cerevisiae* BY4741 mutant strains, one lacking the entire mitochondrial genome (*rho$^0$*), the other lacking cyt c heme lyase, which is essential for the covalent binding of the heme group to apo-cytochrome c (*Δcyc3*). Two deletion strains, *rho$^0$* and *Δcyc3*, and the wild-type cells were treated with 15 or 30 $\mu$M gefitinib for 48 h, and cell numbers were calculated by spotting on a plate containing YPD medium after sequential dilution. The results showed that the two mutant strains were more resistant to death induced by gefitinib, in comparison to the wild-type strain (Fig. 5), thus suggesting that gefitinib induces yeast cell apoptosis via the mitochondrial pathway.

## Discussion

A broad range of stimuli, such as stress conditions and drugs, at a given concentration range, are able to trigger *S. cerevisiae* into the apoptotic process (1,20). In this study, we show that yeast cell death induced by gefitinib, sharing apoptosis features common to those of mammalian, including phosphatidylserine exposure, DNA fragmentation, mitochondrial membrane permeabilization and increased ROS production, is dose-, time- and mitochondrial-dependent.

The contribution of mitochondrial metabolism and biogenesis to yeast apoptosis remains controversial and it has been recently reviewed by Pereira *et al* (19). In this study, we present several results indicating that mitochondrial function is required for cell death induced by gefitinib. Firstly, the reduction of ΔΨ, being accompanied by increased ROS production in yeast cells treated with gefitinib, was detected, thus suggesting that gefitinib treatment causes mitochondrial dysfunctions. Similarly, a decrease of ΔΨ and ROS assessment was observed for yeast apoptosis induced by arsenite (7). Secondly, the strain deleted in cyt c heme lyase (*Δcyc3*) delayed yeast cell death after gefitinib treatment for 48 h, in contrast to the wide-type strain. Cyt c was the first mitochondrial protein with apoptotic function to be identified and hence it established the general importance of mitochondria in apoptosis (21). For acetic acid-induced apoptosis in a mitochondrial-dependent manner, the lack of cyt c was associated to an increased resistance to yeast cell death (22). Thirdly, abrogation of mitochondrial DNA (rho$^0$) improves cell viability during gefitinib treatment, demonstrating a strict requirement of functional mitochondria in gefitinib-induced apoptosis. Actually, rho$^0$ mutants have displayed increased resistance to many apoptotic stimuli, such as acetic acid, arsenic and pheromone (23).

Gefitinib has been used as an effective anti-cancer agent. The mechanisms of its anti-cancer effect are mostly related to the induction of cell apoptosis. For instance, in the human HCC cell lines Huh-7 and HepG2, gefitinib-treatment induced both mitochondrial-dependent and -independent apoptosis. Changes in ΔΨ, caspase-8 activation, caspase-3 activation and nuclear degradation, were also detected. Morevoer, gefitinib suppressed the expression of anti-apoptotic proteins, Bcl-2 and Bcl-$X_L$, further rendering HCC cells prone to apoptosis (11). The present study on gefitinib-induced yeast apoptosis demonstrates a similar mechanism with mammalian cells, although the obvious orthologues of mammalian apoptotic regulators, namely of the Bcl-2 family, are absent in *S. cerevisiae*. Therefore, these simple unicellular eukaryotes could prove to be an attractive system which will be able to elucidate the poorly understood downstream events at the core of apoptotic execution associated with gefitinib.

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