Toxicology Mechanisms and Methods

ISSN: 1537-6516 (Print) 1537-6524 (Online) Journal homepage: http://www.tandfonline.com/loi/itxm20

Dual fluorescent protein-based bioassay system for the detection of genotoxic chemical substances in Saccharomyces cerevisiae

Yixin Lu, Yongjie Tian, Ruikun Wang, Qianqian Wu, Yu Zhang & Xiangming Li

To cite this article: Yixin Lu, Yongjie Tian, Ruikun Wang, Qianqian Wu, Yu Zhang & Xiangming Li (2015): Dual fluorescent protein-based bioassay system for the detection of genotoxic chemical substances in Saccharomyces cerevisiae, Toxicology Mechanisms and Methods

To link to this article: http://dx.doi.org/10.3109/15376516.2015.1070305

Published online: 31 Jul 2015.

Toxicology Mechanisms and Methods

http://informahealthcare.com/txm
ISSN: 1537-6516 (print), 1537-6524 (electronic)

Toxicol Mech Methods, Early Online: 1–10
 DOI: 10.3109/15376516.2015.1070305

Taylor & Francis
Taylor & Francis Group

RESEARCH ARTICLE

Dual fluorescent protein-based bioassay system for the detection of genotoxic chemical substances in *Saccharomyces cerevisiae*

Yixin Lu[1], Yongjie Tian[1], Ruikun Wang[1], Qianqian Wu[1], Yu Zhang[1], and Xiangming Li[1,2]

[1]*Department of Preventive Medicine, Yangzhou Medical College, Yangzhou University, Yangzhou, Jiangsu, PR China and* [2]*Jiangsu Co-Innovation Center for Prevention and Control of Important Animal Infectious Disease and Zoonoses, Yangzhou, PR China*

Abstract

A new reporter system has been developed for quantifying the activity of potentially DNA-damaging substances in the yeast *Saccharomyces cerevisiae*. The system relies on two different reporter genes, *yEGFP* and *DsRed-Express2*, to screen for DNA-damaging chemicals. The *yEGFP* gene is fused to the test promoter of RNR2, whose measurable signal has a dose-dependent relationship with DNA damage. The gene encoding *DsRed-Express2* is fused to a constitutive promoter of GPD, providing an internal control for normalizing cell numbers in the assay. The dual fluorescent protein assay system is performed by sequentially measuring the yEGFP and DsRed-Express2 fluorescent intensity of the same sample, with the results expressed as the ratio of yEGFP to DsRed-Express2 intensity (yEGFP/DsRed-Express2). The yeast fluorescent protein reporter assay was performed in 96-well microtiter plates in the presence of different concentrations of test substances, which were then characterized. The assay was very efficient, high-throughput, and amenable to full automation. Here, we demonstrate that this system can be used as a biosensor to assess the genotoxic potential of drugs and other chemical substances.

Keywords

Chemical substances, DNA damage, DsRed-Express2 reporter, genotoxicity, yeast, yEGFP reporter

History

Received 24 March 2015
Revised 8 May 2015
Accepted 3 July 2015
Published online 31 July 2015

Introduction

There are many agents, both natural and artificial, that cause DNA damage, resulting in many types of DNA damage that affect cells in different ways. To study which chemicals are DNA-damaging agents and gain an understanding of how they function in cells, it is necessary to develop test systems that permit convenient detection and evaluation. In this regard, the development of a genotoxicity test that can detect critical levels of different DNA-damaging substances in environmental samples is of great importance. Over many years, a variety of short-term test systems have been developed using microorganisms, plants, insects, and cultured mammalian cells to screen potentially mutagenic, teratogenic and/or carcinogenic agents. Among these assays, the Ames test is the most widely employed reversion assay system and is performed on Salmonella strains. It is generally robust, economical, and sensitive for evaluating mutagenic activities of chemical agents. The literature suggests that the mutagenic properties of several chemicals correlate well with their carcinogenic effects (Ames, 1979; Ames et al., 1972a,b, 1973a,b, 1975; McCann & Ames, 1976a).

Address for correspondence: Prof. Dr Xiangming Li, Department of Preventive Medicine, Yangzhou Medical College, Yangzhou University, Yangzhou, Jiangsu 225001, PR China. Tel: +86 514 87978855. E-mail: 847264344@qq.com

However, the Ames test is not perfect. *Salmonella typhimurium* is a prokaryote and is, therefore, not a perfect model for humans. There are compounds that are Ames-negative yet lead to cancers in animal models, as well as those that are Ames-positive yet do not pose a threat to humans. These differences are associated with the efficiency of repair and with variations in the metabolism of compounds in the different cell types. So to some extent, whether the data from this biosensor are a reliable source for risk assessment is an ongoing debate (Jia et al., 2002).

Yeasts are eukaryotic cells, and the protein metabolism and DNA repair pathways are the same as in mammalian cells (Luceri et al., 2005). The yeast-based biosensors for DNA-damaging agents consist of two components, the promoter of a DNA damage-responsive gene as the sensor and a reporter. The RNR2 gene is used as a sensor because its expression is nearly undetectable during normal cell growth and is strongly induced after DNA damage (Elledge & Davis, 1968, 1987; Huang & Elledge, 1997). GFP is considered to be a non-invasive reporter of gene expression when illuminated in living cells and can be detected directly; hence, offering an opportunity to develop highly automated live-cell testing systems. Yeast-enhanced GFP (yEGFP) is a codon-optimized GFP in which the nucleotides have been changed to the preferred codon usage for yeast (Billinton et al., 1998; Cormack et al., 1997). The yEGFP assay is sensitive, reproducible, and cheap, which makes it highly suitable for

Abbreviations

4-NQO, 4-nitroquinoline-*N*-oxide
5-FU, 5-Fluorouracil
EB, ethidium bromide
GPD, glyceraldehyde-*p*-dehydrogenase
MMS, methyl methanesulfonate
RNR2, the small subunit of ribonucleotide reductase
yEGFP, yeast enhanced green fluorescence protein

use as a high-throughput system. However, the normalization of yEGFP intensity has routinely been achieved by normalizing for the optical density (A600) of the yeast culture. The use of optical density measurements can yield highly variable results depending on the spectrophotometer and yeast strain. This can add to the experimental inaccuracy of the assay due to variability in the yeast number and the spectrophotometer (McNabb et al., 2005).

In mammalian cells, dual reporters have been commonly used to improve the accuracy of transient-reporter assays when analyzing gene expression. One reporter system used in mammalian cells for gene expression studies is the dual luciferase reporter (DLR) assay (Sherf et al., 1996). However, mammalian cells are highly sensitive to the cell damage induced by toxic agents and extremely sensitive to growth conditions such as equipment nutrient media; furthermore, their biological processes are also more complex. In contrast, the yeast test systems media components are cheaper than those required for mammalian cell systems, and yeast cells grow faster and have increased tolerance of toxic effects such as endotoxins or solvents. Thus, some publications have reported examples of this type of yeast biosensor using the DLR system (Andreotti et al., 2011; Harger & Dinman, 2003; McNabb et al., 2005). Nevertheless, the assays based on dual luciferase reporters use expensive substrates and are labor-intensive due to the need to lyse the cell wall, both of which render the assay unsuitable for high-throughput screening. In contrast, fluorescent reporters do not require substrates and can be detected directly in un-fractionated samples *in vivo* using only excitation and emission wavelengths of light that can be read in photometers.

Therefore, we selected the best combination of yEGFP regulated by the RNR2 promoter and the rapidly maturing red variant DsRed-Express-2 as the two reporters to construct a yeast-based dual fluorescent assay system to screen DNA-damaging chemicals.

Materials and methods

Strains and chemical reagents

The yeast strain used in this study was W303-1A (MATa, ade2-1, his3-11, leu2-3, trp1-1, and ura3-1), which was a gift from Professor Zheng-xiang Wang (the School of Biotechnology, Jiangnan University, Wuxi, China). Transfected yeast strains were grown on the selective yeast media of SD with dropout medium (-trp, -ura, or -ura) obtained from Clontech (Mountain View, CA).

All solvents and chemicals (mutagens) were purchased from Sigma-Aldrich (St. Louis, MO) and the purities are greater than 99%.

The construction of the yeast yEGFP reporter vector regulated by the RNR2 promoter

To generate a pRNR2–yEGFP fusion construct, the RNR2 promoter was amplified by PCR from yeast (W303-1A) genomic DNA. We divided the promoter into two parts. First, we used the forward primer 5′-CATAAGCTTCCGTACCTTC CAGCATTGTCC-3′ containing a restriction site for HindIII (underlined) and the reverse primer 5′-TGCGGATCCCGATA GCGATATGATGAGAAA-3′containing a restriction site for BamHI (underlined) to amplify the RNR2-1, which is about half of the promoter. Then, the next fragment of RNR2-2 which contains a HindIII restriction site was amplified with PCR primers designed with either a BamHI (forward primer) or KpnI (reverse primer) restriction site: forward 5′-TCGGGATCCGCATGGGCGACGAAAAAGCCAA-3′ and reverse 5′-TTTGGTACCGGTAATTGGACAAATAAATAC G-3′. This was performed to avoid introducing a HindIII restriction site on the yeast reporter vector of pLZ-yEGFP (Xu et al., 2010). The touch down PCR program was executed using an ABI-2720 thermocycler (Applied Biosystem, Waltham, MA) using the following conditions: pre-denature template for 5 min at 94 °C; denature the template for 45 s at 94 °C; anneal primers for 45 s at 62 °C; elongate for 1 min at 72 °C; repeat two cycles; then the annealing temperature is gradually decreased 2 °C from 60 °C to 50 °C every two cycles. When the annealing temperature reached 50 °C, 15 cycles were performed. When the cycles were finished, a final extension was performed at 72 °C for 5 min.

The amplified RNR2-1 promoter was purified using the DNA fragment Purification Kit (TaKaRa, Dalian, China), digested with HindIII and BamHI, and then cloned into a HindIII/BamHI-digested pLZ-yEGFP vector to yield pRNR2-1-yEGFP. Also the RNR2-2 was purified, digested with BamHI and KpnI and then inserted into pRNR2-1-yEGFP to yield pRNR2-yEGFP.

The construction of DsRed Express2 yeast express vector

The DsRed Express2 was amplified by PCR from the vector YIPlac204TC-NLS-DsRed-Express2 (from www.addgene.org) using primers forward: 5′-AGAGGATCCATGGATA GCACTGAGAACGTCA-3′ and reverse: 5′-TAGAGAATTC CTACTGGAACAGGTGGTGGCGGGC-3′, which introduce a unique BamHI and EcoRI sites, respectively. The PCR product of DsRed-Express2 was purified and digested with BamHI and EcoRI, and then inserted into the correspondent site of pGPD (Xu et al., 2010) to yield pGPD–DsRed-Express2.

Transformation of yeast

The pRNR2-yEGFP plasmid was transformed into W303-1A yeast cells using the standard lithium acetate method. Transformants were selected on SD/-ura medium lacking uracil. The transformants were then verified by performing

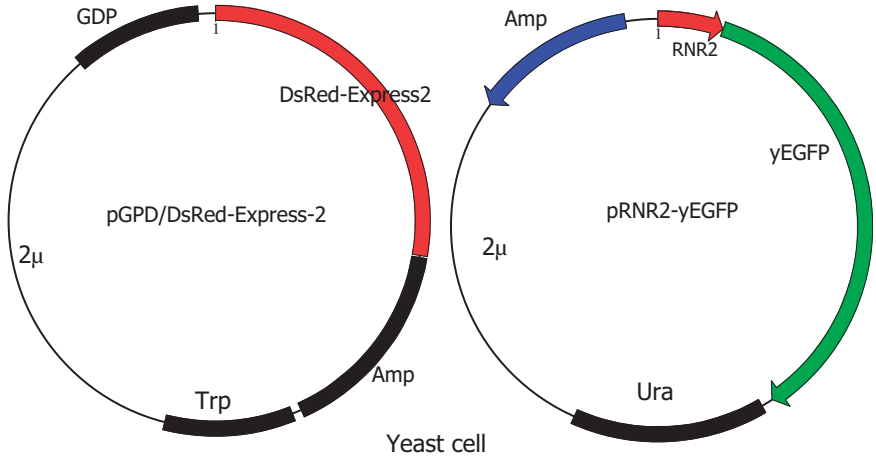

Figure 1. Schematic drawing of the dual fluorescent protein biosensor. The gene encoding DsRed-Express2 is fused to a constitutive promoter, GPD, as a control to normalize the assay for cell numbers. The yEGFP gene is fused to the test promoter, RNR2. yEGFP expression is under the control of the RNR2 promoter, providing a measurable signal that has a dose-effect relationship with some genotoxins.

PCR using RNR2 promoter primers to yield the strain of W303-1A/RNR2-yEGFP.

W303-1A/RNR2-yEGFP was transformed with pGPD-DsRed-Express2. The transformants were selected on SD/-ura, -trp medium lacking uracil and tryptophan and verified by PCR to generate the strain W303-1A/RNR2-yEGFP/DsRed-Express2, which can emit green and red fluorescence (Figure 1).

Dual fluorescence assay

To quantify the biosensor's performance, 96-well black plates were used for the measurement of fluorescence of W303-1A/pRNR2-yEGFP/DsRed-Express2 cells exposed to multiple doses of genotoxins. A single colony of the recombinant yeast was grown overnight at 30 °C with vigorous shaking in 3 mL of the SD/-ura, -trp selective medium. The cell density was diluted with fresh selective medium to a final A600 of 0.1–0.2. Solutions of test substances (2 μL per well) were distributed into the wells in triplicate for each concentration. One hundred microliters of the diluted yeast cell culture was transferred into the wells, resulting in the final test substance concentrations. We added 100 μL of W303-1A/pRNR2-yEGFP/DsRed-Express2 culture and 2.0 μL of water or diluents as a zero concentration reference (blank group). For incubation, plates were sealed with adhesive film, and incubated at 30 °C with shaking. The fluorescence was measured at 0, 4, 8, 12, 16, and 20 h, with $\lambda_{ex} = 485/20$ nm and $\lambda_{em} = 528/20$ nm (green fluorescence), and with $\lambda_{em} = 555/10$ nm and $\lambda_{em} = 585/10$ nm (red fluorescence) using a multi-mode plate reader (Synergy2, Biotek, Winooski, VT).

All substances used are listed and categorized in Table 1. The substances include DNA-binding, -modifying and -leaving molecules, topoisomerase and polymerase inhibitors as well as other biochemically active substances.

Statistical analysis

The green fluorescence intensity was normalized by RFU. The RFU (relative fluorescence unit) = green fluorescence intensity/red fluorescence intensity. The fold induction was calculated using the following formula: fold induction = test chemical RFU/blank RFU. Test compounds with a fold induction greater than 1.5 are considered to be genotoxic.

Table 1. Substances used and test doses.

Tested substances	Dose range
DNA intercalation	
Actinomycin D	0.4–400 μg/L
Ethidium bromide (EB)	0.1–100 μg/mL
Alkylation of DNA	
Mitomycin C	0.05–50 μg/mL
Methyl methanesulfonate (*MMS*)	0.4–400 μg/mL
Chlorambucil	0.2–200 μg/mL
Cleavage of DNA	
cis-Platinum	0.05–50 μg/mL
4-Nitroquinoline-*N*-oxide(4-*N*QO)	0.02–20 μg/mL
Bleomycin	0.05–50 μg/mL
Phleomycin	0.2–200 μg/mL
Inhibitors of polymerases or topoisomerases	
5-Fluorouracil (5-FU)	1–500 μg/mL
Hydroxyurea	0.12–120 mM
Camptothecin	0.06–60 μg/mL
Aphidicolin	0.2–200 μg/mL
Other biochemical activities	
Colchicine	0.5–500 μg/mL
Canavanine	0.2–200 μg/mL
Tetracycline	0.2–200 μg/mL

For each set of replicate concentrations of three wells, the mean value and the standard deviation were calculated. The values of fold-induction were plotted against the substance concentration and incubation time. Error bars show the standard deviation. All calculations and plots were made in Excel 4.0.

Results

W303-1A/RNR2-yEGFP/DsRed-Express-2 cells were treated with different concentrations of test substances of DNA intercalating agents for different lengths of time. Figure 2 shows that actinomycin D began to exhibit a dose-dependent effect at 8 h using the dual fluorescent cytosensor, and the fold induction increased most greatly at 12 and 16 h, with peak induction occurring at the 100 μg/L dose. However, a negative relationship was found at 20 h, perhaps because the actinomycin D became inactivate (Figure 2A). For ethidium bromide (EB), no dose-dependent effects were found in the tested concentration range. However, the fold induction appeared to decrease linearly with the doses at 0 h. This is not due to the expression of DsRed-Express-2 or yEGFP, but

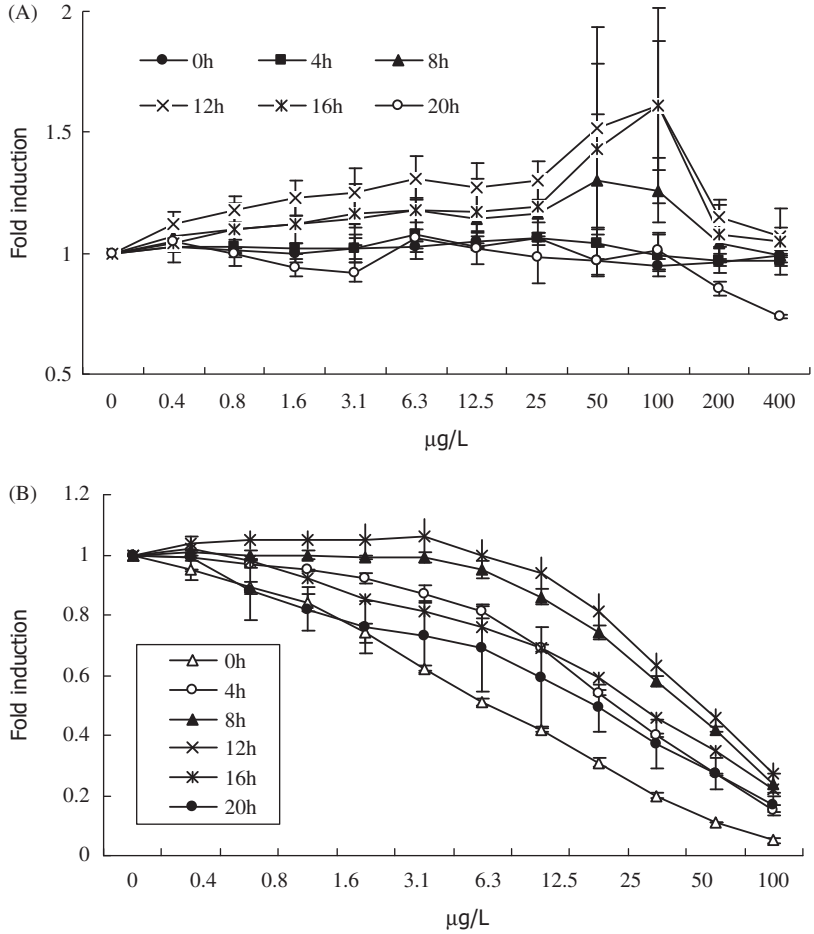

Figure 2. Dose-response curves for DNA intercalating substances. The biosensor was incubated with the test chemicals for different lengths of time. The drug actinomycin D began to exhibit a dose-dependent effect at 8 h and was found to have more than a 1.5-fold induction at 100 µg/L during a 12 and 16 h treatment (A) while EB had no dose-dependent effect (B). For EB, the fold induction appeared to decrease linearly with the doses at 0 h. This is because EB can emit red fluorescence at 590 nm upon light excitation. Over time, RFU increased slowly due to the expression of DsRed-Express-2, but the fold induction did not significantly increase.

rather it was caused by EB, which can emit a red fluorescence of 590 nm upon light excitation. Over time, RFU increased slowly due to the expression of DsRed-Express-2, but fold induction was not significantly increased within the range of tested concentrations (Figure 2B).

Mitomycin C, MMS, and chlorambucil are alkylating agents. As shown in Figure 3, a negative result was observed with mitomycin C. No significant induction in the expression of the yEGFP gene was observed when the cytosensor was treated with mitomycin C in the range of the tested concentration and the exposure time tested and fold induction is close to 1 (in the range of 0.99–1.09) (Figure 3A). However, when MMS and chlorambucil were tested on the yeast cytosensor, the RNR2-yEGFP system was induced. This figure illustrates that the changes in the dose–response curve (Figure 3B) of MMS did not differ substantially from that of chlorambucil (Figure 3C). They both show an obvious dose-dependent effect on the cytosensor at concentrations between 25 and 200 mg/L during a 4 h exposure time. With the prolongation of the exposure time to 8 h, a very strong dose-dependent effect is observed. A distinct induction peak (2.54-fold induction) was found for MMS at concentrations between 25 and 200 mg/L, while the peak fold induction (3.96-fold) for chlorambucil appeared at the 200 mg/L dose.

Representative time and dose-dependent fold inductions are given in response to four DNA cleaving agents: 4-NQO exposure (Figure 4A), *cis*-platinum (Figure 4B), bleomycin (Figure 4C), and phleomycin (Figure 4D). As observed in this figure, the relative induced fluorescence signals correlated with increasing concentrations of the mutagens. For 4-NQO, the time-dependent dose–response was observed at 4 h for 4-h intervals, and a distinct peak was observed at 0.62 mg/L. Although slight increasing changes were observed for 20 h, these were not stable. In contrast, when the cytosensor was treated with *cis*-platinum for 20 h, with bleomycin for 16 h or with phleomycin for 8 h, the dose–response increased linearly. For bleomycin, the fold inductions began to increase with increasing concentrations when the cytosensor was exposed for 4 h. Compared with that of bleomycin, phleomycin only showed dose-dependent effects after 8 h.

As shown in Figure 5, the tested genotoxic chemicals categorized as inhibitors of polymerases or topoisomerases, including 5-fluorouracil (5-FU), hydroxyurea, and camptothecin, all induced the RNR2-yEGFP system to various levels. More than 1.5-fold increases in the fold induction began to be observed for 5-FU at doses of 0.5 µg/mL and greater (Figure 5A), and for camptothecin at doses of 30 mg/L and greater (Figure 5B) when the biosensor was treated with the tested concentrations for 4 h at 4-h intervals, but the dose–response curves increased slowly. However, for hydroxycarbamide, the dose–response curves increased linearly in the tested dose range from 3.75 to 120 mM, although less than a 1.5-fold induction was observed (Figure 5C). However, this reporter system did not respond to aphidicolin at the concentrations tested (Figure 5D).

Some of the tested drugs, including tetracycline, colchicine and canavanine, kill yeast cells without damaging

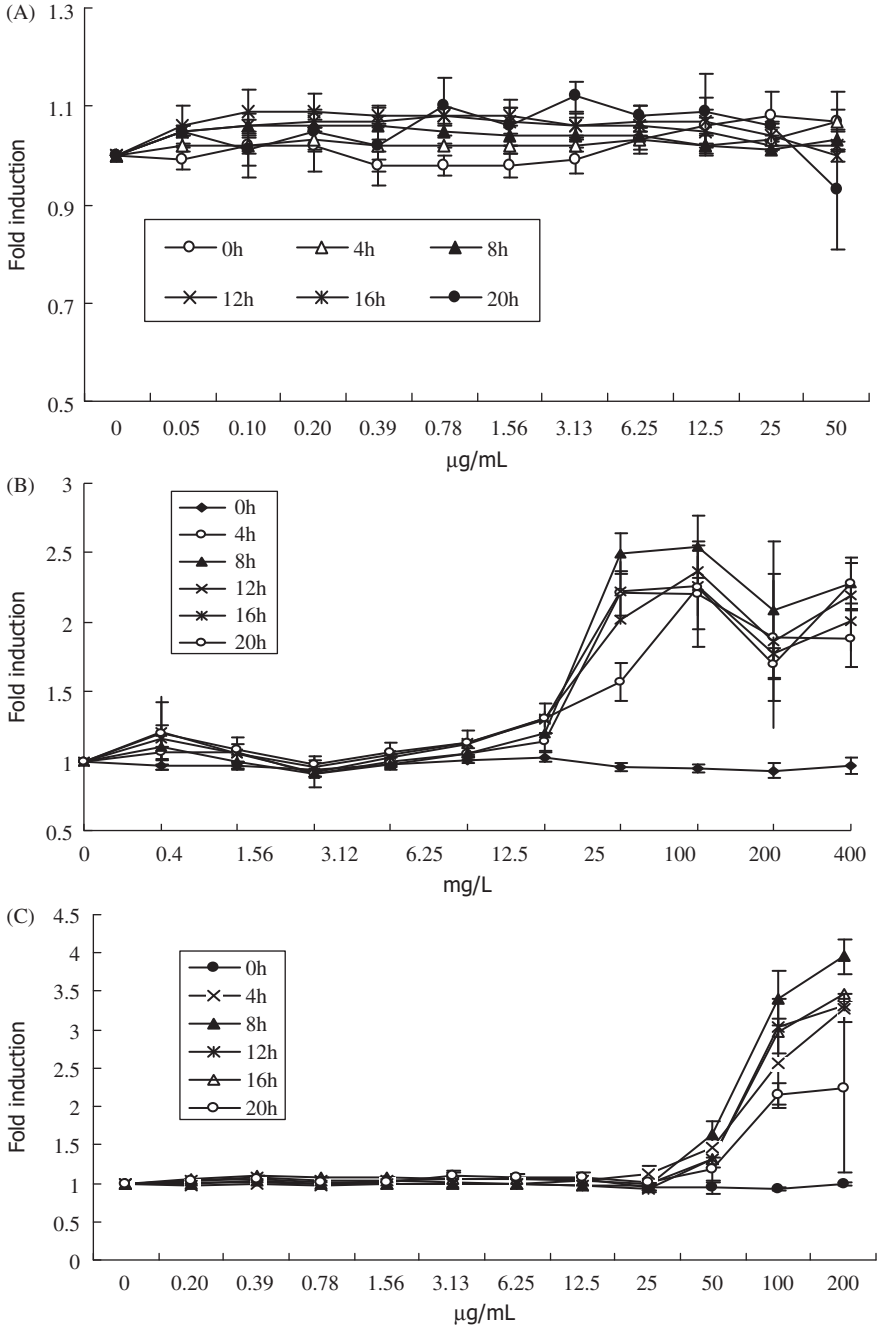

Figure 3. Dose–response curves for DNA alkylating agents. (A) a negative result was observed for mitomycin C, which had a fold induction close to 1; it is possible that the intracellular concentration was not sufficient for activity, or that reduction, a step likely required for activation, was not efficient in yeast. (B and C) Both MMS and chlorambucil had obvious dose-dependent effects with distinct induction peaks of 2.54- and 3.96-fold induction, respectively.

DNA and were unable to induce RNR2-yEGFP expression (Figure 6A–C).

Discussion

DNA damage from chemical substances and carcinogens can be assessed in microorganisms. The Ames test is a widely employed method that uses bacteria to test whether a given chemical is a mutagen, a test that correlates highly with rodent carcinogens (Ames et al., 1972a; 1975; McCann et al., 1975a,b). In general, the test serves as a quick and convenient assay to estimate the carcinogenic potential of a compound because standard carcinogen assays on mice and rats are time-consuming and expensive. To assess the activity of a large number of substances, measurements of toxicity are required that can be fully automated and reliable on a large scale. In addition, the method is less effective in detecting DNA-damaging agents that are mainly toxic but not mutagenic. Moreover, the bacterium *S. typhimurium* is a prokaryote and is, therefore, not a perfect model for humans.

An adapted *in vitro* model has been created for eukaryotic cells, using yeast, for example, in which the organization and regulation of genes is more similar to mammals. Additionally, yeast also has the advantage of being a unicellular microorganism. It grows rapidly and is easy to culture and manipulate such that one can perform a large number of tests in a short period of time (Botstein & Fink, 2011). Several publications have described the development of yeast-based genotoxicity tests in which DNA damage-inducible promoters such as the RAD54, RNR2, or RNR3 gene promoters were fused to reporter genes such as LacZ or the yeast enhanced green fluorescent protein (yEGFP) (Wei et al., 2013;

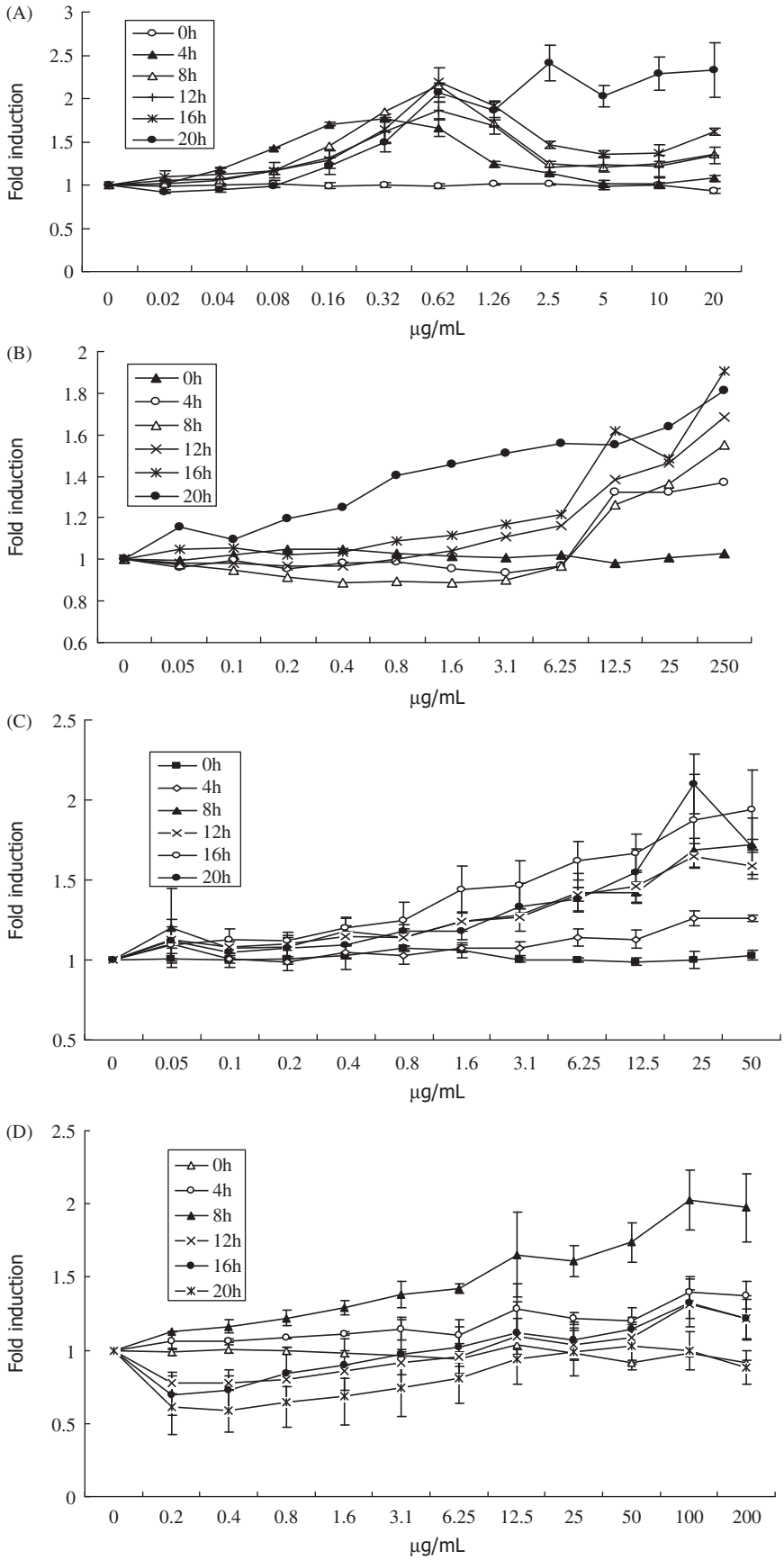

Figure 4. Dose–response curves for DNA cleaving agents. The time-dependent dose responses for the DNA cleaving agents were observed for 4 h at 4-h intervals. A distinct peak in fold induction was observed in response to 4-NQO exposure at 0.62 mg/L (A). However, the dose-dependent effect exhibited increased linearly for a 20 h cis-platinum treatment (B), for 16 h bleomycin treatment (C) and an 8 h phleomycin treatment (D).

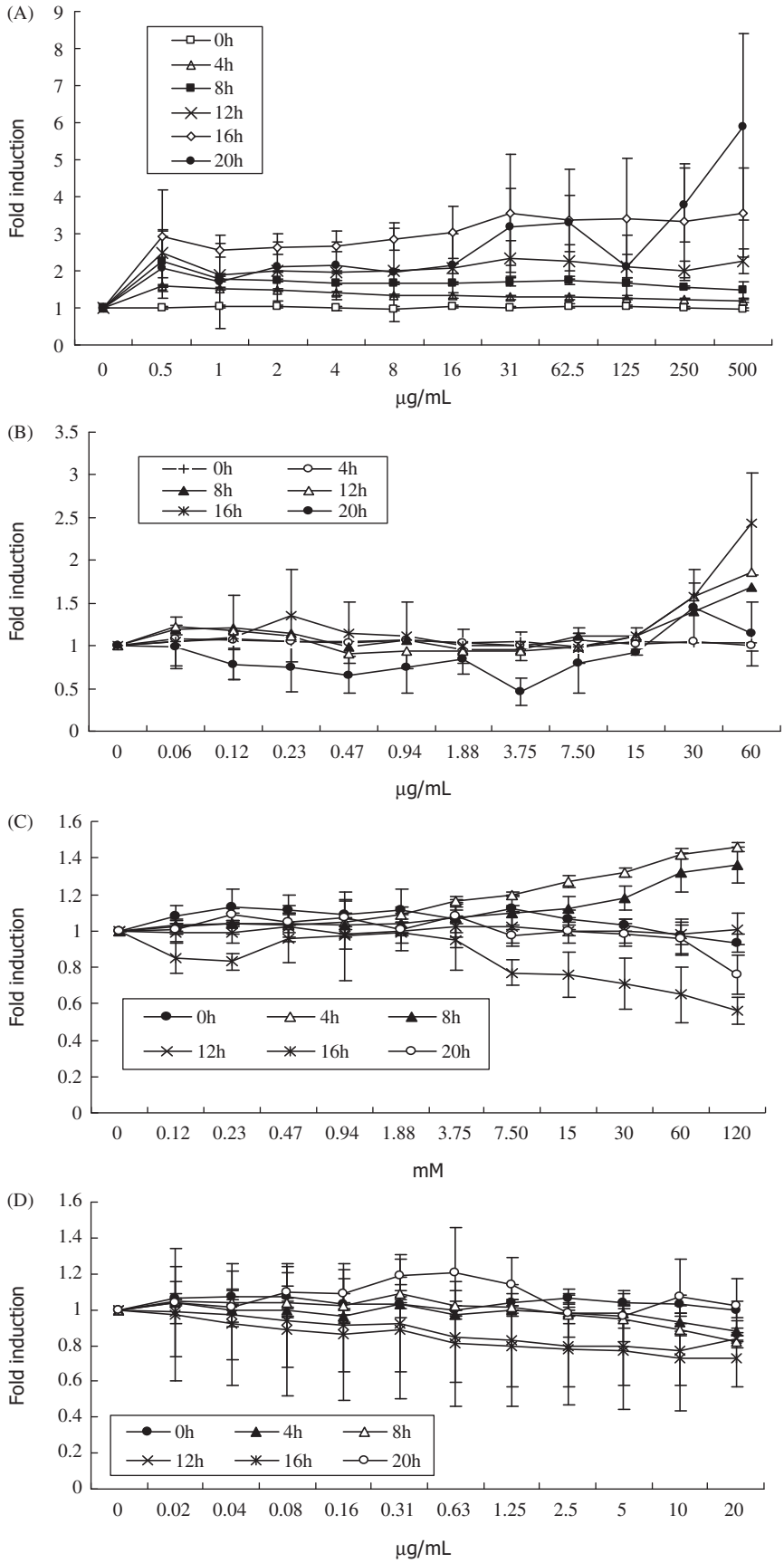

Figure 5. Dose–response curves for inhibitors of polymerases or topoisomerases. More than 1.5-fold increases in the fold induction began to be observed for 5-FU at concentrations of 0.5 µg/ml and greater (A) and for camptothecin at concentrations of 30µg/ml and greater (B) with the curves slowly increasing linearly when the biosensor was treated at the tested concentration for 4 h at 4-h intervals. However, for hydroxycarbamide, the dose-response curves increased linearly in the tested dose range from 3.75 to 120 mM, though less than a 1.5-fold induction was observed (C). However, this reporter system did not respond to aphidicolin at the concentrations tested (D).

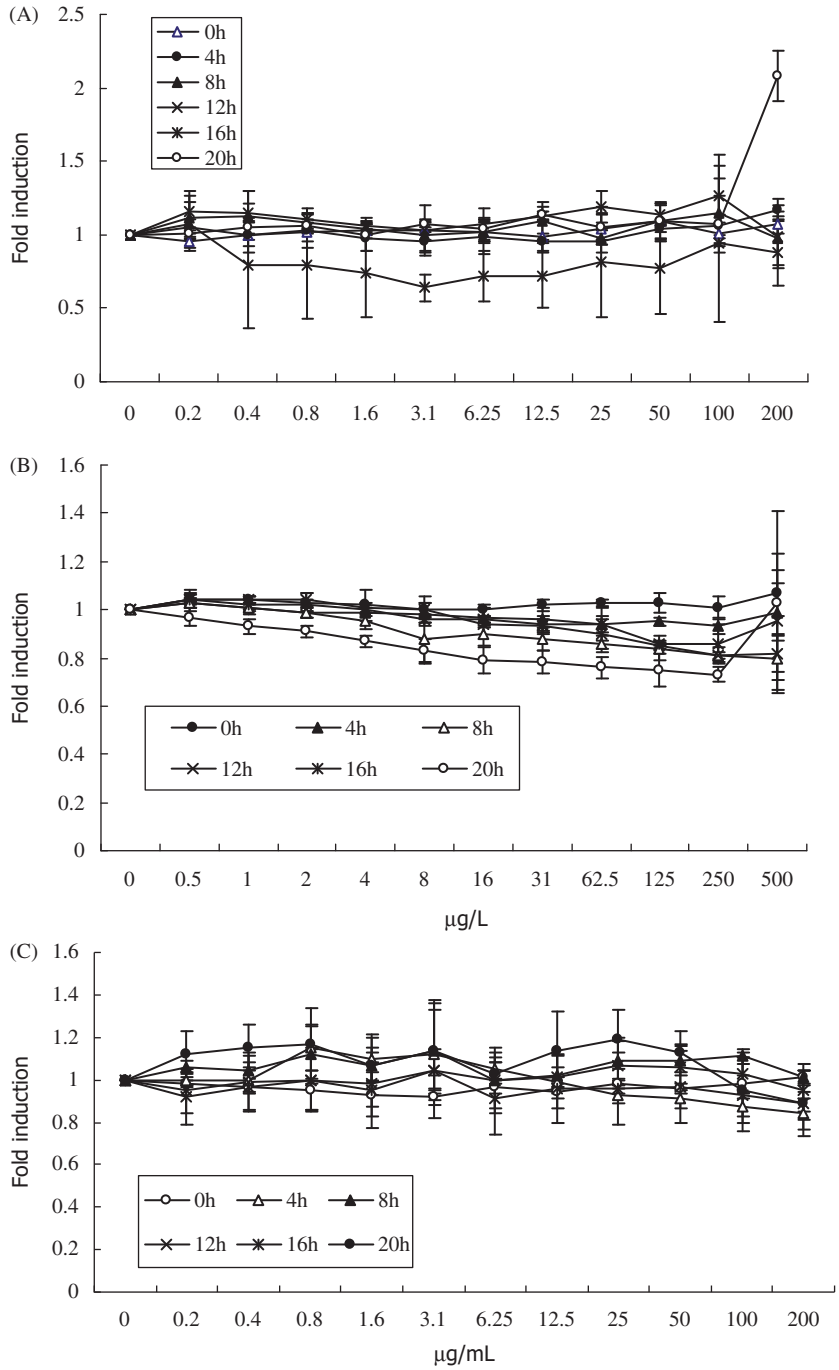

Figure 6. Dose–response curves for the tested drugs that did not damage DNA but showed some cytotoxicity to cells. As shown in the figure, tetracycline (A), colchicine (B) and canavanine (C) were unable to induce RNR2-yEGFP expression.

Zhang et al., 2011). The *lacZ* reporter gene encodes the β-galactosidase whose activity can easily be quantified (Bassaneze et al., 2008; Gary & Kindell, 2005; Itahana et al., 2007).

However, for the determination of enzyme activity, the yeast cell wall has to be disrupted, substrates were added and the enzyme activity was normalized by quantifying total protein of the yeast lysate or yeast density, all of which are time-consuming procedures. The induction of a fluorescence signal driven by promoters that respond to DNA damage in the yeast *S. cerevisiae* provides a simple system to monitor the activity of substances that could possess mutagenic potential at low concentrations. The use of yEGFP, which contains optimal codons for expression in yeast, as a reporter is a useful tool for making the bioassay procedure faster and easier. The microtiter plate format used for incubation and measurement allows simple automation with well-established microtiter plate handling systems used in high-throughput screening.

However, the major drawback of yEGFP assays is that many cellular metabolites and crucial cellular extracts exhibit intense autofluorescence in culture media. Such autofluorescent background can cause experimental inaccuracy. For example, the sensitivity to low concentrations of MMS is not only proportional to MMS concentration but also to the ratio of MMS to cell mass (Afanassiev et al., 2000). Therefore, the normalization of yEGFP assays routinely needs to be achieved on the basis of the optical density (A600) of the yeast culture.

However, the use of optical density measurements can yield highly variable results depending on the spectrophotometer and yeast strain used.

To overcome the drawback of the yEGFP assay, the DLR system had been developed for quantifying gene expression in the yeast *S. cerevisiae*. This system relies on two different reporter genes, Renilla and firefly luciferase, to evaluate regulated gene expression. The gene encoding *Renilla luciferase* is fused to a constitutive promoter as a control for normalizing the assay. The firefly luciferase gene is fused to the test promoter (McNabb et al., 2005; Zhang et al., 2014). However, Renilla and firefly luciferase require costly substrates, and when you determine the luminescence, the cell walls should be disrupted because substrates cannot enter into the cell through the cell wall. Thus, the DLR system is expensive and time consuming.

To avoid the disadvantage of the DLR system, we employed two fluorescent protein genes as reporters, and this dual fluorescent protein assay provides advantages for measuring gene expression in yeast. First, the sensitivity of the yEGFP assay provides a measurable signal that has a dose-dependent response to some mutagens. Second, the use of a constitutive promoter to drive expression of one of the reporters provides an internal control for cell numbers. Third, yEGFP and DsRed-Express2 can be directly observed in living cells over time. Fourth, the DsRed-Express2 fluorescent protein has enhanced solubility, reduced green emission, and faster maturation than the original (Strack et al., 2009, 2011). These advantages allow this bioassay to be performed in a standard black microtiter plate that is fully sufficient for measuring the induction of yEGFP fluorescence driven by the RNR2 promoter and the DsRed-Express2 fluorescence. This microtiter plate format used for incubation and measurement allows simple automation with well-established microtiter plate handling systems used in high throughput screening.

RNR2 is a member of a family of genes with cell-cycle-regulated activities involved in DNA synthesis that are transcriptionally induced in response to the stress of DNA damage. RNR2 is induced in response to a wide variety of agents that either damage DNA directly through chemical modification or induce stress by blocking DNA replication (Elledge & Davis, 1968; Halimi et al., 2011). We have demonstrated in this study that the *RNR2* gene is most likely the best choice among all yeast DNA damage-inducible genes studied to date. *RNR2* not only has one of the highest rates of induction after DNA damage but it is also induced at a much lower dose of DNA damage than most other genes. It is interesting to note that from the two DNA intercalation substances tested, EB and actinomycin D, EB exhibited a negative result that was different from that of Afanassiev's report. This is because this chemical can emit fluorescence at 590 nm.

As expected from the results from DNA-alkylating substances, only the larger molecule mitomycin C was not found to activate the RNR2 promoter driving yEGFP expression. One explanation could be that the intracellular concentration obtained in the yeast was not sufficient for activity, or that reduction, a step most likely required for activation, is not efficient in yeast (Afanassiev et al., 2000).

All the DNA-cleaving substances had clear effects in our assay. Although the unstable increasing changes with 4-NQO treatment were observed for 20 h at the tested concentrations, the fluorescence intensity from DsRed-Express-2 above 1.26 μg/mL was less than that of the blank for 0 h (data not shown). This suggests that the unstable changes with doses are caused by cytotoxic effects rather than genotoxic ones.

Among the four drugs that block topoisomerase I or DNA polymerases, only aphidicolin did not induce fluorescence. This did not agree with an earlier report (Afanassiev et al., 2000). One reason for this discrepancy may be is that our yeast bioassay system is different from the system in previous reports making it difficult to compare them against each other.

We also tested the specificity of our assay using chemicals with other biochemical activities, including colchicines, tetracycline, and canavanine, which are cytotoxic to cells without damaging DNA. The results indicate that these non-genotoxic substances did not cause induction of RNR2.

Acknowledgements

The authors would like to thank Mr. Mingfang Zhang from the Gene Company Limited in China for valuable technical assistance.

Declaration of interest

Part of this work was supported by Ruikun Wang, who is studying for a Master degree in our college. This study was supported by The National Natural Science Foundation of China (No. 81241099) and the Foundation of Practice and Innovation Training Program for Jiangsu College Students (No. B12222).

References

Afanassiev V, Sefton M, Anantachaiyong T, et al. (2000). Application of yeast cells transformed with GFP expression constructs containing the RAD54 or RNR2 promoter as a test for the genotoxic potential of chemical substances. Mutat Res 464:297–308.

Ames BN, Gurney EG, Miller JA, Bartsch H. (1972a). Carcinogens as frameshift mutagens: metabolites and derivatives of 2-acetylaminofluorene and other aromatic amine carcinogens. Proc Natl Acad Sci USA 69:3128–32.

Ames BN, Sims P, Grover PL. (1972b). Epoxides of carcinogenic polycyclic hydrocarbons are frameshift mutagens. Sci New York N.Y 176:47–9.

Ames BN, Durston WE, Yamasaki E, Lee FD. (1973a). Carcinogens are mutagens: a simple test system combining liver homogenates for activation and bacteria for detection. Proc Natl Acad Sci USA 70: 2281–5.

Ames BN, Lee FD, Durston WE. (1973b). An improved bacterial test system for the detection and classification of mutagens and carcinogens. Proc Natl Acad Sci USA 70:782–6.

Ames BN, Mccann J, Yamasaki E. (1975). Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian-microsome mutagenicity test. Mutat Res 31:347–64.

Ames BN. (1979). Identifying environmental chemicals causing mutations and cancer. Sci New York N.Y 204:587–93.

Andreotti V, Ciribilli Y, Monti P, et al. (2011). p53 transactivation and the impact of mutations, cofactors and small molecules using a simplified yeast-based screening system. PLoS One 6:e20643.

Bassaneze V, Miyakawa AA, Krieger JE. (2008). A quantitative chemiluminescent method for studying replicative and stress-induced premature senescence in cell cultures. Anal Biochem 372:198–203.

Billinton N, Barker MG, Michel CE, et al. (1998). Development of a green fluorescent protein reporter for a yeast genotoxicity biosensor. Biosens Bioelectron 13:831–8.

Botstein D, Fink GR. (2011). Yeast: an experimental organism for 21st Century biology. Genetics 189:695–704.

Cormack BP, Bertram G, Egerton M, et al. (1997). Yeast-enhanced green fluorescent protein (yEGFP) a reporter of gene expression in *Candida albicans*. Microbiology 143:303–11.

Elledge SJ, Davis RW. (1968). Identification of the DNA damage-responsive element of RNR2 and evidence that four distinct cellular factors bind it. Mol Cell Biol 9:5373–86.

Elledge SJ, Davis RW. (1987). Identification and isolation of the gene encoding the small subunit of ribonucleotide reductase from Saccharomyces cerevisiae: DNA damage-inducible gene required for mitotic viability. Mol Cell Biol 7:2783–93.

Gary RK, Kindell SM. (2005). Quantitative assay of senescence-associated beta-galactosidase activity in mammalian cell extracts. Anal Biochem 343:329–34.

Halimi Y, Dessau M, Pollak S, et al. (2011). COP9 signalosome subunit 7 from Arabidopsis interacts with and regulates the small subunit of ribonucleotide reductase (RNR2). Plant Mol Biol 77:77–89.

Harger JW, Dinman JD. (2003). An in vivo dual-luciferase assay system for studying translational recoding in the yeast *Saccharomyces cerevisiae*. RNA (New Yorkl, N.Y.) 9:1019–24.

Huang M, Elledge SJ. (1997). Identification of RNR4, encoding a second essential small subunit of ribonucleotide reductase in *Saccharomyces cerevisiae*. Mol Cell Biol 17:6105–13.

Itahana K1, Campisi J, Dimri GP. (2007). Methods to detect biomarkers of cellular senescence: the senescence-associated beta-galactosidase assay. Methods Mol Biol 371:21–31.

Jia X, Zhu Y, Xiao WA. (2002). Stable and sensitive genotoxic testing system based on DNA damage induced gene expression in *Saccharomyces cerevisiae*. Mutat Res 519:83–92.

Luceri C, De Filippo C, Giovannelli L, et al. (2005). Extremely low-frequency electromagnetic fields do not affect DNA damage and gene expression profiles of yeast and human lymphocytes. Radiat Res 164: 277–85.

McCann J, Ames BN. (1976). Detection of carcinogens as mutagens in the *Salmonella*/microsome test: assay of 300 chemicals: discussion. Proc Natl Acad Sci USA 73:950–4.

McCann J, Choi E, Yamasaki E, Ames BN. (1975a). Detection of carcinogens as mutagens in the *Salmonella*/microsome test: assay of 300 chemicals. Proc Natl Acad Sci USA 72:5135–9.

McCann J, Spingarn NE, Kobori J, Ames BN. (1975b). Detection of carcinogens as mutagens: bacterial tester strains with R factor plasmids. Proc Natl Acad Sci USA 72:979–83.

McNabb DS, Reed R, Marciniak RA. (2005). Dual luciferase assay system for rapid assessment of gene expression in *Saccharomyces cerevisiae*. Eukaryotic Cell 4:1539–49.

Sherf BA, Navarro SL, Hannah RR, Wood KV. (1996). Dualluciferase reporter assay: an advanced co-reporter technology integrating firefly and Renilla luciferase assays. Promega Notes 57:2–8. Available from: http://www.promega.com/pnotes/57/5573a/5573a.html.

Strack RL, Hein B, Bhattacharyya D, et al. (2009). A rapidly maturing far-red derivative of DsRed-Express2 for whole-cell labeling. Biochemistry 48:8279–81.

Strack RL, Keenan RJ, Glick BS. (2011). Noncytotoxic DsRed derivatives for whole-cell labeling. Methods Mol Biol 699:355–70.

Wei T, Zhang C, Xu X, et al. (2013). Construction and evaluation of two biosensors based on yeast transcriptional response to genotoxic chemicals. Biosens Bioelectron 44:138–45.

Xu H, Luo F, Zhang J, et al. (2010). Development of a rapid screening and surveillance for estrogenic chemicals in environment based on recombinant yEGFP yeast cell. Toxicol In Vitro 24:1285–91.

Zhang M, Zhang C, Li J, et al. (2011). Inactivation of YAP1 enhances sensitivity of the yeast RNR3-lacZ genotoxicity testing system to a broad range of DNA-damaging agents. Toxicol Sci 120:310–21.

Zhang Q, Lu M, Wang C, et al. (2014). Characterization of estrogen receptor α activities in polychlorinated biphenyls by in vitro dual-luciferase reporter gene assay. Environ Pollut 189:169–75.