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An in vitro system for measuring genotoxicity mediated by human CYP3A4 in *Saccharomyces cerevisiae*

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Abstract

P450 activity is required to metabolically activate many chemical carcinogens, rendering them highly genotoxic. CYP3A4 is the most abundantly expressed P450 enzyme in the liver, accounting for most drug metabolism and constituting 50% of all hepatic P450 activity. CYP3A4 is also expressed in extrahepatic tissues, including the intestine. However, the role of CYP3A4 in activating chemical carcinogens into potent genotoxins is unclear. To facilitate efforts to determine whether CYP3A4, *per se*, can activate carcinogens into potent genotoxins, we expressed human CYP3A4 in the DNA-repair mutant (*rad4 rad51*) strain of budding yeast *Saccharomyces cerevisiae* and tested the novel, recombinant yeast strain for ability to report CYP3A4-mediated genotoxicity of a well-known genotoxin, aflatoxin B1 (AFB₁). Yeast microsomes containing human CYP3A4, but not those that do not contain CYP3A4, were active in hydroxylation of diclofenac (DCF), a known CYP3A4 substrate drug, a result confirming CYP3A4 activity in the recombinant yeast strain. In cells exposed to AFB₁, the expression of CYP3A4 supported DNA adduct formation, chromosome rearrangements, cell death, and expression of the large subunit of ribonucleotide reductase, Rnr3, a marker of DNA damage. Expression of CYP3A4 also conferred sensitivity in *rad4 rad51* mutants exposed to colon carcinogen, 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx). These data confirm the ability of human CYP3A4 to mediate the genotoxicity of AFB₁, and illustrate the usefulness of the CYP3A4-expressing, DNA-repair mutant yeast strain for screening other chemical compounds that are CYP3A4 substrates, for potential genotoxicity.

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AUTHOR CONTRIBUTIONS

Michael Fasullo and Xinxin Ding designed the study. Patricia Egner measured the DNA adducts from yeast strains expressing P450 genes that were exposed to AFB₁. Julian Freedland imaged cells in the Amnis, performed the DCF assays, and assisted with the growth curves. Cinzia Cera verified the presence of CYP3A4 on the expression plasmid using PCR and restriction enzyme digestions. Nick St. John performed the Western blots and growth curves. Matt Hartog performed the HPLC analysis for to detect the CYP3A4 metabolites. All the authors read and approved the study.

Keywords

CYP3A4; budding yeast; Aflatoxin B₁; DNA damage

INTRODUCTION

The incidence of liver cancer strongly correlates with exposure to chemical carcinogens, such as aflatoxin B₁ (AFB₁) (Wild et al., 2009; McGlynn et al., 2011). The correlation between carcinogenic potential and genotoxicity is underscored by observations that a signature p53 mutation is often found in primary liver cancer biopsies from individuals exposed to high levels of AFB₁ (Hsu et al., 1991; Wogan, 1992; Shen and Hong, 1996). These studies indicate that many potent hepatocarcinogens are also potent genotoxins. AFB₁ requires metabolic activation to become a potent genotoxin (Smela et al., 2000). The direct genotoxic AFB₁ metabolite is an epoxy derivative, aflatoxin B₁-8,9-epoxide (AFBO), that reacts with DNA to generate 8,9-dihydro-8-(N⁷-guanyl)-9-hydroxy AFB₁ (AFB₁ N⁷-Guanine) adducts, which spontaneously convert to a AFB₁-formamidopyrimidine (FAPY) derivative and abasic sites (Smela et al., 2000). These DNA adducts are either directly mutagenic or can trigger DNA polymerase arrest, potentially causing chromosomal breakage and subsequent rearrangements. The full genotoxic potential of these DNA adducts is unknown.

Among the 57 human CYPs, 3A4, 2C9, 2C8, 2E1, and 1A2 are well-expressed in the liver, while 3A5, 2A6, 2D6, 2B6, and 2C19 are less abundant (Zanger et al., 2013; Yang et al., 2010). These genes are highly polymorphic and their expression is highly variable among individuals (for review, see Zanger and Schwab, 2013). Both epidemiological and biochemical studies have focused on identifying which CYP450 genes associate with primary liver cancer; however epidemiological studies are inconclusive (Zeigler-Johnson et al., 2004). In vitro experiments have been performed to identify the genotoxic products generated by specific P450 enzymes. For example, CYP1A1-mediated and CYP3A4-mediated metabolism of AFB₁ yields the hydroxylated and less toxic derivatives, aflatoxin M₁ (AFM₁) and aflatoxin Q (AFQ), respectively, while CYP1A2-mediated metabolism of AFB₁ yields a higher proportion of the highly reactive epoxy derivative, AFBO (Neal et al., 1998; Gallagher et al., 1996). CYP1A2 has been alleged to be a major contributor of carcinogen-associated liver cancer because of its higher affinity for AFB₁ (Landi et al., 1999). Other CYPs which metabolize AFB₁ include CYP3A5, CYP2A6, and CYP3A4 (Crespi et al., 1991).

CYP3A4 constitutes 50% of the P450 activity in the human liver (Yang et al., 2010) and is expressed in other tissues exposed to food carcinogens, such as the small intestine (Ding et al., 2003; Gundert-Remy et al., 2014). While CYP3A4 metabolizes many pharmaceuticals, its substrate binding site is wider (Sevrioukova et al., 2015; Guengerich, 2016), rendering a lower affinity for particular compounds. Epidemiological studies are unclear whether CYP3A4 polymorphisms are linked to higher rates of xenobiotic-associated cancer (Klein and Zanger, 2013; Zang and Schwab, 2013). Several studies expressing CYP3A4 and CYP1A2 in lymphocytes or epithelial cells have suggested that CYP3A4-mediated

activation of AFB₁ is less than that of CYP1A2, as measured by p53 mutations or DNA adduct formation (Crespi et al., 1991; Gallagher et al., 1994; Mace' et al., 1997). Considering that AFB₁ is also a potent recombinagen (Sengstag et al., 1994; Sengstag et al., 1996; Keller-Seitz et al., 2004) and can induce a strong DNA damage response (Fasullo et al., 2008), it is important to measure other genotoxic endpoints in measuring the metabolic activation of liver carcinogens.

While both CYP1A2 and CYP3A4 have been expressed in bacteria to measure carcinogen-associated mutations (Kranendonk et al., 1999; Palma et al., 2013), budding yeast are particularly advantageous for measuring multiple genotoxic endpoints, such as checkpoint activation, recombination, mutation, and chromosome missegregation. The one endogenous P450 in budding yeast is insufficient to activate AFB₁ into a potent genotoxin thus allowing for ectopic expression of individual human P450 enzymes (Paladino et al., 1999; Sengstag et al., 1996; Fasullo et al., 2014). Both CYP1A2 (Keller-Seitz et al., 2004) and CYP3A4 (Li et al., 2009) have been individually expressed in yeast; however, mammalian CYP3A4 is particularly prone to proteolysis (Liao et al., 2006). In this study, we individually measured CYP3A4 and CYP1A2-mediated activation of AFB₁ using recombination, growth curves, DNA adducts and Rnr3-GFP assays. Our data indicate that CYP3A4 expression is sufficient to activate AFB₁, resulting in significant lethality in a *rad4 rad51* mutant, defective in both nucleotide excision and recombinational repair. CYP3A4-mediated activation of AFB₁ also stimulates recombination that can result in translocated chromosomes, consistent with the idea that multiple P450 genes can convert AFB₁ into a recombinagen.

MATERIALS AND METHODS

Strains, Media, Chemicals

Yeast strains are listed in Table 1. The human oxidoreductase (hOR) had been introduced in the *TRP1* locus (*trp1::hOR*) as previously described (Fasullo et al., 2014). By genetic crosses, strains were constructed that containing *trp1::hOR* and *his3* recombinational substrates on chromosomes II and IV to measure translocation events (Figure 1; Fasullo and Davis, 1987). The *rad4* strain, defective in nucleotide excision repair (NER), and the *rad4 rad51* strain, defective in both NER and recombinational repair, were also constructed by genetic crosses to contain *trp1::hOR*.

Standard media were used for the culture of yeast and bacterial strains. LB (Luria broth) and LB-AMP (LB containing 100 µg/ml ampicillin) were used for the culture of the bacterial strain DH1 and the DH1 strain containing the vector pMA91-CYP3A4 (Li et al., 2009). Media used for the culture of yeast cells included YPD (yeast extract, peptone, dextrose), SC (synthetic complete, dextrose), SC-LEU (SC lacking leucine), SC-TRP (SC lacking tryptophan), SC-URA (SC lacking uracil), and fluoroarotic acid (FOA) media, as described by Burke et al. (2000). AFB₁ was purchased from Sigma Co., and a 10 mM solution was made in dimethyl sulfoxide (DMSO). The heterocyclic aromatic amine 2-amino-3-methylimidazo[4,5-f]quinolone (IQ) and 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx) were purchased from Santa Cruz Biotechnology and dissolved in methanol and DMSO, respectively.

Plasmids

We obtained the pMA91-CYP3A4 from a yeast strain INV-3A4, described in Li et al (2010). We first extracted total yeast DNA and re-introduced the plasmid into the bacterial strain DH1 by selecting for Amp^R transformants (Ausubel et al, 2000). We confirmed the presence of the CYP3A4 gene by PCR and by mapping DNA endonuclease restriction sites. The forward oligo used to identify CYP3A4 was 5'-TGCCCAGTATGGAGATGTGTTGGT3' and the reverse oligo was 5'-TGCCCAGTATGGAGATGTGTTGGT3'. The pMA91-CYP3A4 plasmids were introduced into yeast strains by selecting for Leu⁺ transformants.

Measuring DNA Damage-Associated Recombination

To measure AFB₁-associated genotoxic events, log phase yeast cells (A₆₀₀ = 0.5–1) were concentrated ten-fold in phosphate-buffered saline (PBS) and exposed to indicated doses of AFB₁, dissolved in DMSO. Cells were maintained in synthetic medium (SC-URA) during the 3h carcinogen exposure. After exposure, cells were washed twice, and an undiluted aliquot was inoculated on SC-HIS to select for His⁺ recombinants. An appropriate dilution was inoculated on YPD to measure viability (Keller-Seitz et al, 2004). Generally, 100–200 colonies are counted to assess viability while 1–20 colonies are counted on SC-HIS plates. Statistical significance was determined by ANOVA and Dunnett's test (Zar, 1996).

Electrophoretic Karyotype of His⁺ Recombinants

Intact yeast chromosomes were prepared as previously described (Fasullo and Davis, 1988). Chromosomal DNA was resolved using a CHEF Mapper XA Chiller clamped homogeneous electrical field (CHEF) electrophoresis system (Bio-Rad). The gels were run at a 60° angle for 18 h with pulse times of 40–75 sec and then for 8 h with pulse times of 110–130 sec. Chromosomal DNA was detected by fluorescence after ethidium bromide staining.

Measuring DNA Adducts

To measure the AFB₁-associated DNA adducts in yeast, we used liquid chromatography–electrospray ionization tandem mass spectrometry (LC-ESI/MS/MS) (Egner et al., 2006). Log phase cultures of yeast expressing human CYP3A4 (pMA91) were exposed to 50 μM AFB₁ for 3 h. Because standard protocols for isolating yeast DNA involve alkaline buffers, rendering the highly unstable AFB₁ N⁷-Gua DNA adducts labile, we have modified the “smash-and-grab” protocol (Hoffman and Winston, 1987) using a neutral buffer containing 10 mM Tris–HCl, 1 mM EDTA, 100 mM NaCl, 2% Triton X-100, 1% SDS, pH 7 (Fasullo et al., 2008). DNA was then isolated from two independent samples of yeast cells. The DNA adducts were identified and measured by high performance liquid chromatography and tandem mass spectroscopy (LC-ESI/MS/MS) after acid hydrolysis.

Growth Assays in 96 Well Plates to Measure AFB₁ Sensitivity

In brief, individual saturated cultures were prepared for each yeast strain. Cell density was adjusted to $\sim 0.8 \times 10^7$ cells/ml for all cultures. We maintained the cells in selective medium (synthetic dextrose lacking leucine, tryptophan, or uracil). In each microtiter well, 95 μl of media and 5 μl of cells (4×10^6 cells) were aliquoted in triplicate for blank, control and experimental samples. For experimental samples, we added AFB₁, dissolved in DMSO, for a

final concentration of 100 nM and 10 μ M. The microtiter dish was placed in a plate reader that is capable of both agitating and incubating the plate at 30°C, as previously described (Fasullo et al., 2010). We measured the OD (A_{660}) at 10 min intervals, for a total period for 22 h, 145 readings. Data at 1h intervals was then plotted. To avoid evaporation during the incubation, the microtiter dishes were sealed with clear optical tape (Fasullo et al., 2010).

Diclofenac (DCF) Activity and Preparation of Yeast Microsomes

Strains were grown to saturation in 20 mL SC-LEU medium. The cells were pelleted, resuspended in 5 ml 50 mM Tris 1 mM EDTA (TE pH 7.4) buffer, incubated at room temperature for 5 min, and suspended in 0.6 M Sorbitol 50 mM Tris (pH 7.4). The cells were lysed by agitation in a bead beater at 4°C, and centrifuged at 10000 $\times$ g for 20 min. The supernatant from each sample was made 0.15 M in NaCl, and 10% in polyethylene glycol (MW 3350), and incubated on ice for at least one hour. Each tube was centrifuged at 10000 $\times$ g for 10 min. The supernatant was removed, and the resulting pellet was suspended in TE and made 20% in glycerol; supernatants were stored in a -80°C freezer. Microsomal extracts were added to 100 μ M diclofenac (DCF), 100 mM KPO_4 , and 1 mM NADPH (Ngui et al., 2000). The assay mixes were then placed in 37°C water bath for 30 min, with minimal light. After the addition of the standards, 0.4 ng DCF-D₄ and 0.4 ng 4'-OH-DCF-¹³C₄, 400 μ l acetonitrile was then added. The samples were centrifuged twice and the supernatants were then analyzed using Ultra High Performance Liquid Chromatography (UPLC), as described (Ngui et al., 2000).

UPLC-MS/MS analysis of the DCF CYP3A4 metabolite, 5'-OH-DCF, was conducted using an Agilent 1290 Infinity liquid chromatography system coupled to an AB SCIEX 6500 QTrap mass spectrometer. Chromatographic separation was performed with an Agilent Eclipse Plus C18 RRHD column (2.1 $\times$ 50 mm; 1.8 μ m) using gradient elution and a 5 μ L sample injection volume. Mobile phase A (A) consisted of 94.9% H₂O, 5% acetonitrile, and 0.1% formic acid and mobile phase B (B) consisted of 94.9% acetonitrile, 5% H₂O, and 0.1% formic acid. The total run time was 6.5 min, with a flow rate of 0.2mL/min, and used the following elution gradient: 95%A for 0.2 min, followed by a linear increase from 5%B to 90%B between 0.2 and 2.0 min, the gradient remained at 90%B until 2.5 min and then returned to 95%A for re-equilibration of the column. The mass spectrometer was operated in the positive mode using electrospray ionization and the following parameters: Curtain gas 30.0, CAD gas medium, ionspray voltage 5500.0, source temperature 375.0°C, source gas 1 35.0, source gas 2 27.0, declustering potential 50.0, exit potential 10.0, collision energy 21.0, collision cell exit potential 11.0. The analyte, 5'-OH-DCF, was detected with multiple reaction monitoring (MRM) using 312.0/230.0 m/z (dwell time: 150 msec) as the parent/product ion transition, 312.0/265.0 m/z was used as a confirmatory parent/product ion transition. Enhanced Product Ion (EPI) scanning was used to generate MS/MS spectra of the CYP3A4 metabolite in order to confirm its identity as 5'-OH-DCF.

Detecting CYP3A4 Expression by Western blots

Expression of CYP3A4 was determined by Western blots. Cells containing CYP3A4 were inoculated in SC-LEU medium. Log phase cells (A_{600} = 0.5–1) were lysed by agitation with glass beads and protein extracts were concentrated by trichloroacetic acid (TCA)

precipitation, as previously described by Foiani et al. (1994). Proteins were separated on 10% acrylamide/0.266% bis-acrylamide gels, and transferred to nitrocellulose membranes. Human CYP3A4 was detected by Western blots using 1:1000 diluted mouse monoclonal anti-CYP3A4 (Invitrogen LSC169171), and a 1:10,000 diluted secondary goat anti-mouse antibody conjugated with horse radish peroxidase (Santa Cruz Biotechnology). Signal was detected by chemiluminescence using ThermoFischer SuperSignal™ West Femto Maximum Sensitivity Substrate.

Measuring *RNR3* Induction by Rnr3-GFP Fluorescence

Cells expressing CYP3A4 were inoculated in SC-LEU medium. Log phase cells ($A_{600}=0.5-1$) were exposed to 50 μM AFB₁ for 3 h, and washed twice in PBS, and then concentrated tenfold. To measure *RNR3*-GFP expression after radiation exposure, we used an X-ray radiation source purchased from Faxitron, Inc. (Wheeling, IL), and the dose rate was 100 rad/min. Approximately 10e4 cells were then analyzed in the Amnis Image Stream. Cell shape and GFP fluorescence was measured with both a visible light and a 495 nm laser, using the image stream software to calibrate cell shape and fluorescence. Untreated cells were used as controls.

RESULTS

CYP3A4 Activity and Protein Can Be Detected in Yeast

To confirm that the recombinant yeast strain expresses functional human CYP3A4, we performed CYP3A4 activity assays using DCF as the substrate. Microsomal preparations from a strain (YB226) expressing CYP3A4 were compared to a strain not expressing CYP3A4. The results showed CYP3A4-dependent formation of 4'-OH-DCF, a known DCF metabolite produced by CYP3A4 metabolism (Figure 2, panels A, C). In positive control experiments, 4'-OH-DCF was also detected in reactions containing rat liver post-mitochondrial S9 fraction as a source of P450 enzymes (Figure 2B). Formation of the 5'-OH-DCF metabolite using the yeast microsomes is 0.88 pmol/min*mg protein, N = 3. These results confirm that YB226 cells express functional CYP3A4 enzyme.

We detected CYP3A4 protein in yeast cells (YB226) by Western blot using a mouse monoclonal antibody made against rat CYP3A4 (Supplemental Figure). Cell extracts were obtained from control cells lacking the pMA91-CYP3A4 expression vector and cells expressing CYP3A4. Protein was extracted, TCA-precipitated, resolved on SDS-PAGE gels, and blotted on nitrocellulose paper. Yeast protein was visualized by Coomassie staining, and CYP3A4 was identified by 50 kDa protein standard, consistent with the size reported by the commercial supplier (Invitrogen).

Expression of CYP3A4 in the DNA-repair Mutant, *rad4 rad51*, Confers Sensitivity to AFB₁

Inhibition of growth in the *rad4 rad51* strain is indicative of genotoxicity, since this strain is defective in both nucleotide excision and recombinational repair (Fasullo et al, 2010). We observed that growth of CYP1A2-expressing *rad4 rad51* cells was inhibited after exposure to 1 μM AFB₁, while exposure to higher concentrations largely blocked growth, consistent with previous studies (Figure 3; Fasullo et al., 2010). Similarly, we exposed CYP3A4-

expressing *rad4 rad51* cells to AFB₁ and observed growth inhibition after exposure to 100 nM AFB₁ (Figure 3). Growth inhibition was not evident until 7–10 h, the time at which cells enter exponential phase; previous results indicate that AFB₁ elicits a strong DNA damage response as cells enter S phase (Fasullo et al., 2010). By measuring area under the curve, growth was inhibited by $(44 \pm 4)\%$ and $(48 \pm 0.4)\%$ when CYP1A2-expressing cells and CYP3A4-expressing cells were exposed to 20 μ M AFB₁, respectively. The *rad4 rad51* cells not expressing any exogenous CYP did not exhibit sensitivity to 1 μ M AFB₁ (Figure 4). These studies indicate that CYP3A4 metabolically activates AFB₁ into a DNA damaging agent.

CYP3A4 Expression in Yeast Increases AFB₁-associated Recombination

We had previously shown that either CYP1A2 or CYP1A1 expression in yeast is sufficient to metabolically activate AFB₁ into a potent recombinagen (Sengstag et al., 1996; Keller-Seitz et al., 2004). Considering that growth of a CYP3A4-expressing *rad4 rad51* mutant, which is defective in homologous recombination, is inhibited after exposure to AFB₁, we hypothesized that expression of CYP3A4 in yeast is also sufficient to activate AFB₁ into a recombinagen. We introduced pMA91-CYP3A4 into a *rad4* diploid strain that can measure translocation events (Figure 1) and is defective in nucleotide excision repair (NER); NER mutants exhibit enhanced levels of recombination likely due to an inability to remove AFB₁-associated DNA adducts (Keller-Seitz et al., 2004). We exposed the CYP3A4-expressing yeast to 25 μ M, 50 μ M, 100 μ M AFB₁, and measured the frequencies of AFB₁-associated recombination (Figure 5). We also compared the frequencies of AFB₁-associated recombination in cells expressing either CYP1A2 or CYP3A4 after exposure to 50 μ M AFB₁ (Table 2). The overall spontaneous frequency of recombination was higher in cells expressing CYP3A4, compared to the cells expressing CYP1A2 (Table 2). Significant levels of AFB₁-associated recombination were observed after cells were exposed to 50 μ M AFB₁ (Figure 5, $P < 0.05$). In cells exposed to 50 μ M AFB₁, there was a 56% reduction in viability and a six-fold increase in recombination ($N=3$). In comparison, in cells expressing CYP1A2 that were exposed to 50 μ M AFB₁, there was a 49% reduction in viability and more than a twenty-fold increase in recombination; the higher fold increase is due to the lower spontaneous recombination frequency. Significant levels of AFB₁-associated recombination was not observed after CYP3A4-expressing cells were exposed 100 μ M AFB₁, likely due to low viability ($< 10\%$) after exposure. Although a direct comparison of the enzymatic activities of CYP1A2 and CYP3A4 would require additional experiments, we conclude that expression CYP3A4 in yeast is sufficient activate AFB₁ into a potent recombinagen.

To verify that His⁺ recombinants contain chromosomal translocations (Figure 1), we characterized the electrophoretic karyotype of ten AFB₁-associated His⁺ recombinants, using pulse field gel electrophoresis. We expected to observe reciprocal translocations, *CEN4::II* and *CEN2::IV*, where *HIS3* is present on *CEN2::IV*, as previously documented (Sengstag et al., 1996; Fasullo and Davis, 1988). Reciprocal translocations were present in 3/10 of the recombinants, and we speculate that 7/10 of the recombinants lost *CEN4::II*, although it is also possible that the altered mobility was due to additional rearrangements. These results confirm that AFB₁ can stimulate homologous recombination in CYP3A4-expressing yeast, generating chromosomal rearrangements.

AFB₁ N⁷-Guanine DNA Adducts are Detected in Yeast Cells Expressing CYP3A4

Since AFB₁-associated recombination was detected in CYP3A4-expressing yeast cells, we hypothesized that AFB₁-associated DNA adducts should also be detected. Diploid cells expressing either CYP3A4 or CYP1A2 were exposed to 50 µM AFB₁, lysed, the DNA extracted, and the AFB₁ N⁷-guanine DNA adducts were measured (Table II). No AFB₁ N⁷-guanine DNA adducts were detected in cells exposed to DMSO. Overall levels of DNA adducts in cells expressing CYP3A4 were similar to that observed previously in cells expressing CYP1A2 after exposure to AFB₁ (Fasullo et al., 2008; Fasullo et al., 2014). These results indicate that, similar to CYP1A2, expression of CYP3A4 induces AFB₁ N⁷-guanine DNA adducts.

Expression of CYP3A4 in Yeast Stimulates *RNR3*-GFP Expression After AFB₁ Exposure

RNR3 encodes a subunit of ribonucleotide reductase that is DNA damage-inducible and has a very low expression in the absence of DNA damage (Elledge and Davis 1990). Expression generally peaks about three h after exposure. We detected Rnr3-GFP expression in cells using the Amnis flow cytometer in CYP1A2 and CYP3A4-expressing cells after exposure to AFB₁. We detected fluorescence in ~30% of the cells expressing CYP1A2 (n=2, ~6,000 cells). As can be seen in Figure 6, among CYP3A4-expressing cells, both small and large-budded (dumb-bell cells) were fluorescent; the number of which were significantly different ($P < 0.05$) than those treated with DMSO alone ($P < 0.05$). We conclude that CYP3A4-mediated activation of AFB₁ triggered a DNA damage response.

Activation of heterocyclic aromatic amines (HAAs) by expression of CYP3A4 in yeast

CYP3A4 is also expressed in the colon (Ding and Kaminsky, 2003) and could potentially activate heterocyclic aromatic amines that are present in ingested food. We therefore exposed a CYP3A4-expressing *rad4 rad51* strain to IQ and MeIQx, which are potent colon carcinogens. We detected growth inhibition when CYP3A4-expressing cells were exposed to either 150 µM IQ or MeIQx; IQ exposure conferred 21% growth inhibition, while 1.5 mM MeIQx exposure conferred 15% growth inhibition (Figure 4). MeIQx exposure did not inhibit growth in cells that did not express CYP3A4, but some growth inhibition was observed when cells were exposed to IQ.

DISCUSSION

CYP3A4 importance in drug metabolism is well-established. While CYP3A4 can accommodate diverse substrates due to large binding site (Yano et al., 2004), other CYPs, such as CYP1A2, have narrower binding site rendering a greater specificity for particular carcinogens, such as AFB₁ and heterocyclic aromatic amines (HAAs) (Guengerich et al., 2016). It is unclear whether CYP3A4-mediated carcinogen metabolism can elicit similar genotoxic responses as that mediated by CYP1A2. We expressed CYP3A4 in budding yeast and measured genotoxic endpoints after AFB₁ exposure, including DNA adduct formation, recombination, *RNR3*-GFP induction and growth inhibition of a DNA repair mutant. The major conclusion of this manuscript is that CYP3A4 expression in yeast is sufficient to activate AFB₁ into a genotoxin and recombinagen. We suggest that CYP3A4-mediated activation of AFB₁ in humans could also result in a recombinagen.

Our previous studies indicated that CYP1A2-mediated activation of AFB₁ elicits a robust DNA damage response in yeast as measured by AFB₁-associated recombination and the growth inhibition of the *rad4 rad51* DNA repair double mutant. Although we did not directly compare the intracellular activity of either CYP3A4 or CYP1A2, AFB₁-associated genotoxicity phenotypes are as readily detectable in yeast strains that express CYP3A4 as those that express CYP1A2. Here, we reported lower AFB₁-associated recombination frequencies and DNA adduct concentrations in cells expressing CYP1A2 than in previous studies (Fasullo et al., 2008, Fasullo et al., 2014). However, in the strains used in this study, hOR was expressed at a chromosomal location and was not over-expressed on a multi-copied vector. Further experiments are in progress to determine whether CYP3A4-mediated activation of AFB₁ also triggers other genotoxic endpoints, such as Rad53 activation, which are observed after CYP1A2-mediated activation (Fasullo et al., 2008, Fasullo et al., 2010).

Our data indicate that CYP3A4 expression in yeast is sufficient to activate AFB₁ into a DNA damaging agent at concentrations as low as 100 nM. Other biomarkers in CYP3A4-expressing yeast, such as α -amylase inhibition, have been detected by other investigators after exposure to lower AFB₁ concentrations (Li et al., 2009). Previous studies indicate that CYP3A4 likely generates ten-times more AFQ than AFBO (Gallagher et al., 1994). Thus it would be important to know whether different AFB₁ metabolites affect different cellular functions, such as RNA translation and protein stability.

Although CYP3A4-mediated activation of AFB₁ may be most relevant to hepatocarcinogenesis, the expression of CYP3A4 in the human intestine suggests that the gut may be prone to carcinogen-induced toxicity, as induced by AFB₁ and HAAs. We did not measure HAA-associated recombination because co-expression of NAT2 is required to obtain detectable levels of carcinogen-associated recombination (Paladino et al., 1999). Studies done in rat indicate that acute exposures of AFB₁ result in minimal toxicity, perhaps due to the intestinal microbes that bind AFB₁ adducts. Further studies are necessary to know the fate of the AFB₁-associated metabolites in the gut (Wang et al., 2016).

In conclusion, we have shown that CYP3A4 expression in yeast is sufficient to induce DNA damage and elicit genotoxic responses in yeast. These studies allow us to determine whether other carcinogens can be activated in yeast by expression of CYP3A4 and open the avenue for studying CYP3A4 polymorphisms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Configuration of *his3* fragments in yeast strains to measure translocations. Figure on the left is a model of the recombination assay and the figure on the right is a pulse field gel of chromosomal DNA obtained from AFB₁-associated His⁺ recombinants. The oval represents a centromere and the line represents the chromosome; the left arm of the chromosome is not shown for simplicity. The *his3* fragment is shown with arrow and feathers. The similar shaded areas represent shared homology; the tightly-spaced diagonals represent 146 bp of shared DNA sequence and speckles represent 300 bp of shared DNA sequence. An “X” denotes where a cross-over event would occur. The product of the recombination event is shown below where *CEN2* is linked to the long arm of chromosome IV (*CEN2::IV*) and *CEN4* is linked to the long arm of chromosome II (*CEN4::II*). The translocated chromosome *CEN4::II* is identified as a novel band on the pulse field gel, while the translocated chromosome *CEN2::IV*, IV, and XII often comigrate. Lanes A and B are His⁺ recombinants; Lane C is the His⁻ parent. Chromosomal sizes indicated on the left are based on New England Biolab yeast chromosome pulse field standards.

Figure 2.

Results of DCF assays. Reaction mixtures contained (A) microsomal extracts from *rad4 rad51* strain expressing no CYPs (YB226, 0.5 mg protein/ml), (B) rat liver S9 fraction (0.5 mg protein/ml), or (C) microsomal extracts from *rad4 rad51* expressing CYP3A4 (YB439, 0.5 mg protein/ml); 100 μ M DCF; 100 mM KPO₄ buffer, pH 7.4; and 1 mM NADPH, in 1.0 ml final volume. The incubation was performed at 37°C for 30 min, and terminated by the addition of 400 μ l of acetonitrile, containing internal standards. Equal amounts (200 μ l) of the extracted samples (from A–C) were analyzed by LC-MS, as described in Methods. (D), LC-MS chromatogram of standard 4'-OH-DCF-¹³C₆ showing retention time of 2.82 min, the same as shown for the metabolite peak in B and C (arrow).

Figure 3.

Growth curves of the DNA repair *rad4 rad51* mutant expressing CYP1A2 (YB404) or CYP3A4 (YB439) after exposure to AFB₁. The figure represents one experiment where each data point is the average of two biological duplicates. Approximately 10^5 cells were inoculated in each well in a 96-well plate platform; yeast growth in each well was monitored in a Tecan Plate reader and over a period of twenty-four hours. Absorbance (A_{600}) is plotted against time (h). The first panel is growth curves of cells expressing CYP1A2 after exposure DMSO or indicated doses of AFB₁. The second panel is growth curves of cells expressing CYP3A4 after exposure DMSO or indicated doses of AFB₁. Error bars are standard deviations of two biological duplicates.

Figure 4.

Growth curves of the DNA repair *rad4 rad51* mutant expressing CYP3A4 (YB439) or no CYP after exposure to AFB₁, MeIQx, or IQ. Approximately 10⁵ cells were inoculated in each well in a 96-well plate platform; yeast growth in each well was monitored in a Tecan Plate reader and over a period of twenty-four hours. Absorbance (A₆₀₀) is plotted against time (h). The first row is growth curves of cells expressing CYP3A4 or no CYP after exposure DMSO or indicated doses of AFB₁. The second row is growth curves of cells expressing CYP3A4 or no CYP after exposure DMSO or indicated doses of MeIQx. The third row is growth curves of cells expressing CYP3A4 or no CYP after exposure DMSO or indicated dose of IQ. Error bars are standard deviations of two biological duplicates.

Figure 5.

Recombination frequencies and viability after cells expressing CYP3A4 are exposed to AFB₁. Approximately 10⁸ cells on SC-HIS plates to select for His⁺ recombinants, and the appropriate dilution was plated on YPD to measure viability. (Left) Recombination frequencies are represented in a bar graph for each exposure to AFB₁ (0 μM, 25 μM, 50 μM, 100 μM). (Right) Percent viability is represented in a bar graph for each exposure (0 μM, 25 μM, 50 μM, 100 μM). Significant levels of AFB₁-associated recombination were observed after cells were exposed to 50 μM AFB₁ as determined by ANOVA ($P = 0.04$) and Dunnett's test ($P < 0.05$).

Figure 6. The DNA damage response in CYP3A4-expressing cells (YB440) measured by *RNR3* induction. The yeast cells containing *RNR3*-GFP and expressing CYP3A4 were exposed to either DMSO, 2 Krad X-rays, or 50 μM AFB₁. GFP fluorescence of single cells was measured in the Amnis. The percent cells showing significant GFP fluorescence was plotted against each type of exposure. The percent of fluorescent cells is indicated for the single unbudded cells and large budded (G2-arrested) cells.

Table 1

Yeast Strains

Strain (Synonym)	Genotype	Autonomous plasmid	Reference (Source)
YA101 (MCY776)	<i>MATa ura3-52 his3- 200 ade2-101 lys2-801</i>		M. Carlson
BY4741	<i>MATa ura3 0 leu2 0 his3 1 LYS2 met15 0</i>		wiki.yeastgenome.org/index.php/Commonly_used_strains
YB225	<i>MATa-inc ura3 rad4::KanMX</i>		This laboratory
YA283	<i>MATa ura3 0 leu2 0 his3 1 LYS2 met15 0 RNR3-GFP</i>		wiki.yeastgenome.org/index.php/Commonly_used_strains, derived from BY4741
YB226	<i>MATa-inc tpt1 ura3 his3 ade2 rad4::KanMX rad51::URA3</i>		This laboratory
YB400	<i>MATa-inc tpt1 ura3 his3 ade2 rad4::KanMX rad51</i>		Derived from YB226
YB404	<i>MATa-inc tpt1 ura3 his3 ade2 rad4::KanMX rad51</i>	pCS316	This laboratory, Ura ⁺ transformant of YB400
YB318	<i>MATa ura3-52 his3- 200 ade2-n tpt1- 1 gal3- leu2-3, 112 GAL1::his3- 5' tpt1::his3- 3'::HOcs</i>		This Laboratory
YB407	<i>MATa ura3-52, tpt1::hOR his3- 200, ade2-101 lys2-801, leu2</i>		This Laboratory
YB425	<i>MATa ura3-52 his3- 200 ade2-n tpt1- 1 gal3- leu2-3, 112 GAL1::his3- 5' tpt1::his3- 3'::HOcs rad4::kanMX</i>		Derived from cross of YB318 and YB225
YB426	<i>MATa ura3-52, tpt1::hOR his3- 200, ade2-101 lys2-801 rad4::KanMX</i>		Derived from cross of YB407 and YB225
YB427	<i>MATa/MATa ura3-52/- his3- 200/- ade2-101/ade2-n tpt1- 1 tpt1::hOR leu2-3, 112/leu2-801 GAL1::his3- 5' tpt1::his3- 3'::HOcs rad4::kanMX/ rad4 kanMX</i>		Diploid cross of YB425 and YB426
YB428	<i>MATa/MATa ura3-52/- his3- 200/- ade2-101/ade2-n tpt1- 1 tpt1::hOR leu2-3, 112/leu2-801 GAL1::his3- 5' tpt1::his3- 3'::HOcs rad4::kanMX/ rad4 kanMX</i>	pRS424 CYP1A2	Trp ⁺ transformant of YB427
YB437	<i>MATa/MATa ura3-52/- his3- 200/- ade2-101/ade2-n tpt1- 1 tpt1::hOR leu2-3, 112/leu2-801 GAL1::his3- 5' tpt1::his3- 3'::HOcs rad4::kanMX/ rad4 kanMX</i>	pMA91-CYP3A4	Leu ⁺ transformant of YB427
YB438	<i>MATa ura3 leu2 tpt1::hOR rad4::KanMX rad51 his3- 200</i>		Derived from cross of YB400 and YB407
YB439	<i>MATa ura3 leu2 tpt1::hOR rad4::KanMX rad51</i>	pMA91-CYP3A4	Leu ⁺ transformant of YB438
YB440	<i>MATa/MATa ura3 0/ura3-52 leu2 0/leu2 tpt1::hOR/+ his3 1/his3- 200 met15 0/MET RNR3-GFP</i>	pMA91-CYP3A4	Derived from cross of YB439 and YA283

AFB₁ DNA adducts and translocation frequencies in *rad4* mutants expressing CYP1A2 or CYP3A4

Table II

Strain ^a	Plasmid	Spont. Frequency (x 10 ⁻⁷)	Induced Frequency ^b (x 10 ⁻⁷)	Viability ^c	Fold induction ^d	AFB ₁ DNA adducts (pmol/mg DNA) ^e
YB428	pRS424-CYP1A2	0.5 ± 0.2	13 ± 7	51%	29	3.4
YB437	pMA91-CYP3A4	1.6 ± 1.3	21 ± 14	44%	5.6	26.2

^aFor complete genotype, see Table I

^bAfter exposure to 50 µM AFB₁, His⁺ colonies/Total colony forming unit (CFU), N = 2

^cCFU after exposure to AFB₁ / CFU without exposure × 100%

^dFrequency after AFB₁ exposure (Induced Frequency)/Frequency before exposure (Spont. Frequency)

^eN = 2