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Abstract

UDP-glucuronosyltransferase proteins (UGT) catalyze the glucuronidation of both endogenous and xenobiotic compounds. In previous studies UGT1A1 has been implicated in the detoxification of certain food-borne-carcinogenic-heterocyclic amines. To determine the importance of UDP-glucuronosyltransferase 1A1 (UGT1A1) in the biotransformation of the cooked-food carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), genetically modified CHO cells that are nucleotide excision repair-deficient, and express cytochrome P4501A2 (UV5P3 cell line) were transfected with a cDNA plasmid of human UGT1A1 to establish the UDP-glucuronosyltransferase 1A1 expressing 5P3hUGT1A1 cell line. Expression of the UGT1A1 gene was verified by screening *neo* gene expressing clonal isolates (G-418 resistant) for their sensitivity to cell killing from PhIP exposure. Five of eleven clones were chosen for further analysis due to their resistance to cell killing. Western blot analysis was used to confirm the presence of the UGT1A1 and CYP1A2 proteins. All five clones displayed a 52 kDa protein band, which corresponded to a UGT1A1 control protein. Only four of the clones had a protein band that corresponded to the CYP1A2 control protein. Correct fragment size of the cDNAs in the remaining 4 clones was confirmed by RT-PCR and quantification of the mRNA product was accomplished by real-time RT-PCR. Expression of UGT1A1 in the transfected cells was 10^4-10^5 fold higher relative to the UV5P3 parental cells. One clone (#14) had a 10 fold higher increase in expression at 1.47×10^5 over the other three clones. This clone was also the most active in converting *N*-hydroxy-PhIP to *N*-hydroxy-PhIP glucuronide conjugates in

microsomal metabolism assays. Based on the D_{50} values, the cytotoxic effect of PhIP was decreased ~350 fold in the 5P3hUGT1A1 cells compared to the UV5P3 control cells. In addition no significant increase in mutation frequency was observed in the transfected cells. These results clearly indicate that UGT1A1 plays a critical role in PhIP biotransformation, providing protection against PhIP mediated cytotoxicity and mutagenicity.

1. Introduction

Exposure to heterocyclic amines (HAs) in the diet is rapidly becoming an important risk factor in the etiology of certain human cancers. These compounds are most commonly found in well-done cooked meats at the ppb level. All the compounds thus far tested are mutagenic in the Ames/*Salmonella* assay and carcinogenic in rodent bioassays [1]. In humans, epidemiology studies have indicated an increase risk of colon tumors associated with HA exposure from well-done red meat consumption [2]. 2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) is the most mass abundant HA found in cooked beef and chicken. PhIP causes mammary, colon and prostate tumors in rats [3,4] and lymphomas in mice [5]. PhIP has also been shown to form DNA adducts in several human tissues [6,7]. In addition, intake of PhIP from well-done red meat has been associated with an increased risk for breast cancer in women [8].

The bioactivation of PhIP is highly dependent on the cytochrome P4501A2-mediated *N*-hydroxylation to the corresponding 2-hydroxyamino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (*N*-hydroxy-PhIP) [9,10]. Subsequent esterification by sulfotransferase and/or acetyltransferase can generate highly electrophilic *O*-sulfonyl and *O*-acetyl esters, respectively, that can bind DNA [11]. Alternatively, UDP-glucuronosyltransferase mediated glucuronidation of *N*-hydroxy-PhIP results in the formation of the less reactive *N*-hydroxy-PhIP-N^2-glucuronide and *N*-hydroxy-PhIP-*N*3-glucuronide, which can be excreted through urine or bile. It has been proposed that competition between glucuronidation and esterification can determine the susceptibility

to PhIP mutagenicity, and ultimately cancer. In humans, glucuronidation of *N*-hydroxy-PhIP is a major pathway in the biotransformation of PhIP [12]. Furthermore, a recent study has shown that in microsomal preparations, human UDP-glucuronosyltransferase 1A1 (UGT1A1) is the primary enzyme responsible for *N*-hydroxy-PhIP glucuronidation [13]. To further study this important pathway, Chinese hamster ovary (CHO) cells have been engineered to express the human UGT1A1 gene so that its role can be better understood while studying its genotoxic impact.

Previous investigations have studied PhIP bioactivation using the nucleotide repair-deficient CHO UV5 cell line transfected with cytochrome P4501A2 (UV5P3), and *N*-acetyltransferase or sulfotransferase cDNAs [14-16]. In this report, to study PhIP glucuronidation, cDNA from human UGT1A1 was transfected into the CHO UV5P3 cells. With this cell line, the effect of UGT1A1-mediated glucuronidation on PhIP induced cytotoxicity, metabolism, and mutagenesis was assessed. The establishment of this new cell line will help understand the role this enzyme plays in the metabolism of PhIP.

2. Materials and Methods

2.1. Chemicals

PhIP was purchased from Toronto Research Chemicals (North York, Ontario, Canada). *N*-hydroxy-PhIP was obtained from SRI International (Palo Alto, CA, USA). The purity of each chemical was checked by HPLC (isocratic at 40% methanol) and was

determined to be greater than 97% pure. UDP-glucuronic acid (UDPGA) was purchased from Sigma (St. Louis, MO, USA). All immunoblotting reagents were obtained from BioRad, (Hercules, CA.). Antibodies for immunoblotting were obtained from BD Gentest (Bedford, MA) and Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). All other reagents were of analytical grade or better.

2.2. Cell lines

The CHO parental cell line AA8, which is heterozygous at the *aprt* locus [17] was used to derive the UV5 cell line[18], which lacks nucleotide excision repair due to a mutation in the XPD (ERCC2) gene [19]. Transfection of the UV5 cells with mouse cytochrome P4501A2 (CYP1A2) cDNA established the UV5P3 line [14]. The UV5P3 cell line was used for the transfection of the human UDP-glucuronosyltransferase 1A1 (UGT1A1) cDNA, and resulting in cell line 5P3hUGT1A1. All cells were cultured in α-MEM with 10% fetal bovine serum, 100 μg of streptomycin per ml of media, and 100 U of penicillin per ml of media.

2.3. Construction of UGT1A1 expression plasmid

The cDNA of human UGT1A1 was cloned into Xho1 and Xba1 restriction sites of the mammalian expression plasmid pcDNA3 (Invitrogen, Carlsbad, CA). To generate the UGT1A1 cDNA, the pBluescript-UGT plasmid (kind gift of Thomas R. Tephly, University of Iowa) was digested with Xho1 and Xba1 to release the 1840-bp fragment. The excised gel-separated 1840-bp fragment was

purified using QIAEX11 gel extraction kit (Qiagen, Valencia, CA) and ligated into the similarly digested and dephosphorylated pcDNA3.

2.4. Transfection of UV5P3 and selection of transformed clones

Exponentially growing UV5P3 cells were transfected with pcDNA3-UGT1A1 plasmid by electroporation with a Cell-Porator Electroporation System following the protocol supplied by the manufacturer (Life Technologies, Rockville, MD). UV5P3 cells at a density of $1x10^7$ were electroporated with 10 μg of the plasmid cDNA. The transfected cells were plated onto a 100-mm dish with 20 ml α-MEM and incubated for 24 h. The media was then changed to 10 ml of α-MEM containing 1.6 mg/ml of the compound G-418 sulfate (Geneticin) (Life Technologies, Rochville, MD) for selection of clones expressing the *neo* gene. G-418 resistant colonies arose at a frequency of 3.6×10^{-3}. The isolated colonies were removed and transferred to larger culture vessels for expansion.

2.5. Determination of endogenous intracellular glucuronic acid

The UV5P3 parental cell line was analyzed for intracellular glucuronic acid, which is a necessary cofactor for the glucuronosyltransferase reaction to proceed. The presence of glucuronic acid in the UV5P3 cells was determined by the method of Vilim [20]. Cell extracts from the UV5P3 cells contained 1.33 μg of glucuronic acid/mg of protein. Due to this small amount of glucuronic acid in the UV5P3 cells, an external supply of 15mM of UDPGA was added for all the cellular reactions.

2.6. Verification of the UGT1A1 gene in the UV5P3 transformants

To verify the presence of the UGT1A1 gene in the newly transformed cells, eleven clonal isolates were screened for their sensitivity to cell killing from PhIP exposure. 3000 cells were seeded in 96-well plates containing 200 μl of complete α-MEM media and 15 mM UDPGA. Different concentrations of PhIP were added to the clonal isolates and the parental line UV5P3 (in quadruplets), and incubated for 48 h until confluency was reached in the control samples. Quantification of cell proliferation was achieved by following the colorimetric CellTiter 96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI). Quantification of the metabolically active cells was achieved by measuring the absorbance at 490 nm.

2.7. Detection of UGT1A1 and CYP1A2 proteins by immunoblotting

Immunoblot analysis was used to confirm the presence of UGT1A1 and CYP1A2 protein in five (5P3hUGT1A1-3, -5, -7, -12, -14) out of the eleven clonal isolates. Immunoblotting was performed according to the manufacturers recommendations (BD Gentest). Briefly, approximately 50-100 μg of microsomal protein from each 5P3hUGT1A1 clone was heated at 95°C for 4 min in loading buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 25% glycerol, 0.01% Bromophenol Blue) containing 2% 2-mercaptoethanol, and then separated on a 10% SDS-polyacrylamide gel. The separated proteins were electrotransferred onto a nitrocellulose membrane. The membrane was blocked in 5% nonfat powdered milk in 25 mM Tris-HCl (pH 7.5), 150 mM NaCl (solution A) for 1 h, and then washed three times with solution A containing 0.1% Tween 20. Membranes were then incubated for 1 h in 0.5% nonfat powdered milk in solution A

containing an antibody prepared from a rabbit immunized with a peptide specific for human UGT1A1 (WB-UGT1A1; BD Gentest) or a primary goat polyclonal CYP1A2 IgG antibody (Santa Cruz Biotechnology, Inc.). The membrane was washed three more times and was then incubated for 1 h in 0.5% nonfat powdered milk in solution A containing an HRP-conjugated goat anti-rabbit IgG secondary antibody for UGT1A1 detection, or a rabbit anti-goat IgG-HRP for CYP1A2 detection, followed by three more washings. Visualization was performed using Immun-Star HP substrate detection kit (BioRad, Hercules, CA). UGT1A1 and CYP1A2 protein content was compared to known UGT1A1 and CYP1A2 protein standards.

2.8. RT-PCR and real-time RT-PCR

Total RNA from the newly developed cell lines of 5P3HUGT1A1-5, -7, -12, -14 and the parental UV5P3 line was isolated by following the method of RNeasy from Qiagen. For RT-PCR, oligonucleotide primer pairs were synthesized by Gynosys (Woodlands, Texas) and consisted of UGT1A1-653F (tgctcattgccttttcacag) and UGT1A1-891R (ggcttcaaattcctgggatag) which are within the coding region of exon 1 of the UGT1A1 gene. Traditional RT-PCR was performed to determine the size and integrity of the amplified fragment. Both cDNA and PCR were performed in a single tube by following the protocol of the SuperScript One-Step RT-PCR system (Life Technologies, Rockville, MD). The 241 bp PCR product was analyzed by agarose gel electrophoresis.

Real-time RT-PCR was done using SYBR Green chemistry (Molecular Probes, Eugene, OR) for the detection of the PCR products. The primer pairs used were the same that was used for the traditional RT-PCR (UGT1A1-653F and UGT1A1-891R). Quantification of the mRNA products was

accomplished using the comparative C_T (threshold cycle) method. The C_T values of the target UGT1A1 gene in the newly engineered cell lines were compared to the C_T values from the control UV5P3 cell line. All the C_T values were normalized to the endogenous GAPDH housekeeping gene, thus allowing quantification of the mRNA target. This gene was chosen because the amplification efficiency of the UGT1A1 target and the GAPDH gene was similar. Briefly, the First-Strand cDNA was synthesized from the total RNA of the newly engineered and parental cell lines by following the protocol of SuperScript™ II Rnase H Reverse Transcriptase (Invitrogen, Carlsbad, CA). The SYBR Green Master Mix, (Applied Biosystems, Foster City, Ca) was used in the second step of the two-step RT-PCR protocol which contained AmpliTaq Gold DNA Polymerase. The reaction profile consisted of a 15 min incubation at 95 °C for one cycle, followed by 15 sec at 95°, then 60 sec at 58° C for 40 cycles. An ABI Prism 7700 sequence detection system (Applied Biosystems) was used to run the reactions. Data analysis software from ABI Prism 7700 Sequence Detection System (Applied Biosystems) was used to quantitate the target amplicon relative to the calibrator (UV5P3) based on the mathematical formula $2^{-\Delta\Delta CT}$ (User Bulletin #2, ABI Prism 7700 Sequence Detection System, Applied Biosystems).

2.9. CHO cell microsomal metabolism

500 ml of cells were grown to confluency in α-MEM. The cells were pelleted by centrifugation and washed two times in ice-cold PBS. The cells were then homogenized in ice-cold PBS using a Teflon-glass homogenizer until lysis was complete as assessed by light microscopy. The cell lysate was centrifuged at 9000 x g for 20 min at 4°C. The supernatant was removed and centrifuged at 100,000 x g for 60 min at 4°C. The microsomal pellet was resuspended in 50 mM Tris-HCl, pH 7.5 and the protein

concentration was determined using the Bradford assay [21]. Microsomal incubations were prepared on ice in 1.5 ml conical plastic tubes and consisted of 1-5 mg/ml microsomal protein, 8.0 mM $MgCl_2$, 0.5 mM EDTA, 2.0 mM UDPGA, 25 μg/ml alamethicin, and 100 μM PhIP or 25 μM *N*-hydroxy-PhIP (dissolved in DMSO delivered in 5 μl) in 50 mM Tris-HCl buffer, pH 7.5 in a total volume of 200 μl. After a 3 h incubation, 2 volumes of ice-cold methanol were added to each sample to precipitate the proteins and terminate the reaction. The samples were then allowed to stand at -20° C for 30 min. The protein was then removed by centrifugation in a microcentrifuge at maximum speed for 5 minutes. The methanolic extracts containing the reaction products were placed in clean plastic tubes and stored at -80°C until HPLC analysis.

2.10. HPLC analysis of glucuronide conjugates

The aqueous-methanol extracts from the microsomal incubations were evaporated to dryness under nitrogen, then reconstituted in 60 μl of HPLC starting mobile phase. The samples were centrifuged in a microcentrifuge for 1 min at maximum speed and 50 μl of supernatant was injected into an Alliance HPLC system (Waters Corp., Milford, MA) equipped with a 5 μm, 4.6 X 150 mm TSK-GEL ODS-80 TM column (Toso Bioscience, Montgomeryville, PA) and a Waters 990 photodiode array detector. Metabolites were eluted at 0.75 ml/min using a gradient starting at 30% methanol/0.1% triethylamine, pH 6.0, up to 55% methanol/0.1% triethylamine, pH 6.0, at 8 min. The methanol concentration was maintained at 55% from 8 to 20 min. The identities of the *N*-hydroxy-PhIP-glucuronide conjugates were confirmed by comparing the HPLC

retention time and UV spectra to known metabolite standards, and quantified based on the molar extinction coefficient of PhIP (19440 $mol^{-1}Lcm^{-1}$ at 315 nm).

2.11. Assays for cytotoxicity and mutation at the aprt locus

PhIP was added in a dose dependent fashion, to exponentially growing cells in T25 culture flasks containing 1 x 10^6 cells in 10 ml of α-MEM media and 15 mM UDPGA. After 48 h of PhIP exposure, the cells were rinsed twice with PBS and removed with trypsin. Survival dishes and 40 ml suspension cultures at 2.5 x 10^4 cells per ml were initiated. The relative survival value was calculated by determining the ratio of colony-forming units after PhIP exposure relative to colony forming units for the control samples containing dimethylsulfoxide (DMSO) vehicle. After a 3-day phenotypic expression period, cultures were plated for cloning efficiency and mutation. *Aprt* mutants were selected in media containing 8-azaadenine (80 μg/ml) and 10% (v/v) dialyzed fetal bovine serum. 8-Azaadenine dishes were seeded with 6 x 10^5 cells/100-mm dish (six replicates) and incubated at 37°C for 10-13 days; cloning efficiency dishes were seeded with 300 cells/100 mm dish (triplicate) and incubated for 8 days.

3. Results

G-418 resistant clones were isolated and expanded for further analysis. Expression of the UGT1A1 gene was inferred using the non-radioactive cell proliferation assay provided by Promega. Sensitivity to PhIP was evaluated on each clonal isolate and compared to the UV5P3 parental cell line. Addition of PhIP to incubations containing

UV5P3 cells caused a steep concentration dependent decline in cell proliferation. Five of the 11 G-418 resistant clones tested displayed no significant change in cell proliferation after PhIP exposure (Figure 1). Another four clones displayed cell proliferation very similar to the UV5P3 cells, and the other two clones displayed an intermediate level of proliferation. Clones 3, 5, 7, 12, and 14 displayed the greatest amount of protection against PhIP induced cytotoxicity, and were presumed candidates for expressed UGT1A1. In the UV5P3 cells, at a concentration of 20 μg/ml PhIP, the cell survival fraction was reduced to 0.25 relative to the samples containing 0 μg/ml PhIP, whereas, in the 5 candidate clones no change in cell survival was seen. The protection against cytotoxicity observed in the five clonal isolates only suggests there was an increase in PhIP detoxification from the formation of glucuronide conjugates due to the presence and functionality of the UGT1A1 gene. However, genetic manipulations could also be responsible for some unknown lose in gene expression that could alter cell proliferation. Further investigations, described below, were performed to confirm this result.

The presence of the UGT1A1 gene in the five clones that showed the greatest decrease in cell killing was confirmed by Western blot analysis. Each clone displayed a protein band with a molecular mass of approximately 52 kDa which corresponded to the UGT1A1 control protein (data not shown). Interestingly, the UV5P3 parental cell line also produced a faint protein band at the same molecular mass indicating some endogenous UGT1A1 activity in these cells. Preliminary cell proliferation screening showed that the addition of 15 mM UDPGA to the UV5P3 cells, that had been exposed to 10 μg/ml PhIP, increased cell proliferation by approximately 40% over UV5P3 cells without UDPGA.

To ensure the transformed cells were still fully capable of converting PhIP to the N-hydroxy-PhIP intermediate (a possible explanation for decreased cell proliferation), each of the 5 clones was assayed for the presence of the CYP1A2 protein by Western blot analysis. All the clones displayed a protein band that corresponded to the CYP1A2 control protein except clonal isolate 5P3hUGT1A1-3 (data not shown). This clone was subsequently omitted from further analysis.

After 40 cycles of RT-PCR amplification, all the clones including the parental UV5P3 cells expressed the 241 bp fragment of the UGT1A1 gene (Figure 2). However, the amount expressed in UV5P3 was ~100 fold less than the newly engineered cell lines (5P3hUGT1A1-5, -7, -12, -14). In order to further validate and quantitate the mRNA products, real-time RT-PCR was performed. Real-time RT-PCR analysis revealed a 10^4 -10^5 fold increase in UGT1A1 expression, in all the transfected cells tested, over the UV5P3 parental cell line (Table 1). The 5P3hUGT1A1-14 clonal isolate had the highest fold increase in the expression of the UGT1A1 gene at 1.47×10^5 relative to the control UV5P3 cell line. The other isolates had expression levels 10 fold lower than that of the 5P3hUGT1A1-14 cells. Quantification of the mRNA products was accomplished using the comparative C_T (threshold cycle) method. The C_T values of the target UGT1A1 gene in the newly engineered cell lines were compared to the C_T values from the control UV5P3 cell line. All the C_T values were normalized to the endogenous GAPDH housekeeping gene, thus allowing quantification of the mRNA target.

To determine the capability of the CHO cells transfected with the UGT1A1 gene to form glucuronide conjugates, microsomes were prepared from the different clones and exposed to PhIP or *N*-hydroxy-PhIP. When exposed to *N*-hydroxy-PhIP, microsomes

from 5P3hUGT1A1-5, -7, -12, and -14 had detectable activity toward forming the *N*-hydroxy-PhIP- glucuronides with 5P3hUGT1A1-14 being the most active (thus confirming the gene expression data), forming 17 pmol and 1.3 pmol of *N*-hydroxy-PhIP-N^2-glucuronide and *N*-hydroxy-PhIP-*N*3-glucuronide, respectively (Table 2). All the clones tested had detectable levels of *N*-hydroxy-PhIP-N^2-glucuronide, whereas only 5P3hUGT1A1-14 produced *N*-hydroxy-PhIP-*N*3-glucuronide. Microsomes prepared from 5P3hUGT1A1-14 showed a protein concentration dependent increase in conjugate formation (Figure 3). The amount of *N*-hydroxy-PhIP-N^2-glucuronide produced was ~5 times greater than the amount of *N*-hydroxy-PhIP-*N*3-glucuronide. This ratio was consistent at all protein concentrations tested. No glucuronide conjugates could be detected from the microsomes that were exposed to PhIP. PhIP was, however, converted to very small amounts (pmol quantities, determined by HPLC) of *N*-hydroxy-PhIP indicating cytochrome P450 activity is present in these cells (data not shown). The levels of *N*-hydroxy-PhIP were presumably so low that any glucuronides produced would be below the limits of detection. Since 5P3hUGT1A1-14 was the most active in forming *N*-hydroxy-PhIP glucuronides it was investigated further to confirm the presence and the impact of glucuronide activity in these cells.

The ability of PhIP to produce cell killing and mutations at the *aprt* locus (assessed by resistance to 8-azaadendine) was evaluated in both UV5P3 and 5P3hUGT1A1-14 cells. In the UV5P3 cells, PhIP produced a concentration dependent increase in cell killing over a PhIP concentration range of 0.01-0.35 μg/ml (Figure 4a). Based on the D_{50} values (PhIP dose at 50% cell survival) the cytotoxic effect from PhIP was ~350-fold higher in the UV5P3 parental cells compared to the 5P3hUGT1A1-14

cells. The dramatic increase in cytotoxicity in the UV5P3 cells was accompanied by a mutation frequency of 23 per 10^5 viable cells at 0.35 μg/ml PhIP (figure 4b). At the same PhIP concentration no significant increase in mutation frequency was observed in the 5P3hUGT1A1-14 cells. A slight increase in mutation frequency was observed at a PhIP concentration of 25 μg/ml, followed by a decline as the PhIP concentration increased. This decline was presumably due to conversion of damaged cells to those with lethal lesions.

4. Discussion

The introduction of the human UGT1A1 cDNA into the CYP1A2-expressing UV5P3 cells has allowed for the assessment of UGT mediated metabolism of the cooked-food carcinogen PhIP in an intact cellular system. Since UGT1A1-mediated glucuronidation is a detoxification pathway for PhIP, it was hypothesized that CHO cells transfected with the UGT1A1 cDNA should display protection against the cytotoxic effects of PhIP, when screened in the cell proliferation assay. Of the eleven clonal isolates tested only five displayed significant protection against PhIP cytotoxicity. Even though the other six clones were resistant to G-418 (indicating successful transfection of the cDNA) four showed cell proliferation similar to the UV5P3 parental cell line, and the other 2 displayed intermediate activity. This reduced cell survival could be due to a loss in functionality of the newly incorporated UGT1A1 gene or other changes in gene expression. The five clones which displayed cell proliferation similar to incubations containing 0 μg/ml PhIP (5P3hUGT1A1-3, -5, -7, -12, and -14) were, therefore, selected

for further testing to confirm the presence of a functional UGT1A1 gene. The presence of the UGT1A1 as well as CYP1A2 in the 5 clonal isolates was confirmed by Western blot analysis. The lack of CYP1A2 in the 5P3hUGT1A1-3 clone excluded it from further analysis since this isolate would be incapable of hydroxylating PhIP to *N*-hydroxy-PhIP; a critical first step in *N*-glucuronidation. It is interesting to note that Western blot analysis revealed the presence of UGT1A1 in the UV5P3 parental cell line, albeit at levels approximately 100 times lower than the transfected cells. The addition of UDPGA to these cells increased cell proliferation over the UV5P3 cells without UDPGA by approximately 40% at a PhIP dose of 10 μg/ml. Since the UGT1A1 levels were so low compared to the cells that were transfected with the UGT1A1 cDNA it was concluded that the UV5P3 cells would still be a suitable control for the experiments.

The remaining four clones were subjected to real-time RT-PCR in an effort to quantify the expression of the UGT1A1 mRNA in each clone. Clone 5P3hUGT1A1-14 had ten times more mRNA expression than the other three UGT1A1 expressing clones, relative to the UV5P3 parental cells. This clone was also the most active in the microsomal metabolism studies producing both *N*-hydroxy-PhIP-N^2-glucuronide and *N*-hydroxy-PhIP-*N*3-glucuronide. The five times more *N*-hydroxy-PhIP-N^2-glucuronide produced over *N*-hydroxy-PhIP-*N*3-glucuronide was half of what was reported in an earlier study [13]. The difference in this ratio could be due to the use of different host expression systems between the two studies. Both of these metabolites have also been detected in human urine from individuals that have been exposed to PhIP [12,22]. The lack of detectable *N*-hydroxy-PhIP-*N*3-glucuronide from the 5P3hUGT1A1-5, -7, and -12 microsomal preparations was presumably due to low UGT1A1 activity. Based on these

experiments, clonal isolate 5P3hUGT1A1-14 was designated as 5P3hUGT1A1, a viable CYP1A2, UGT1A1 expressing repair-deficient CHO cell line.

Establishment of 5P3hUGT1A1 has made it possible to study the effect UGT1A1 has on PhIP-induced mutagenicity and cytotoxicity. The reduction in mutation frequency and the increase in cell survival compared to the UV5P3 parental cell line clearly indicates that UGT1A1-mediated metabolism of PhIP plays a significant role in PhIP biotransformation. These results are in accordance with previous studies showing UGT1A1 as the primary enzyme involved in *N*-hydroxy-PhIP *N*-glucuronidation [13]. The protection against mutagenicity and cell killing provided by UGT1A1 may play a critical role in determining the susceptibility to the carcinogenic potential of PhIP.

UGT1A1 is one of the most prevalent UGT isozymes found in the body, it is differentially expressed in many different tissues, and plays an important role in the metabolism of both endogenous and exogenous compounds (reviewed in [23]). Polymorphic expression of UGT1A1 has been well documented [24-28], and reports have shown that 7-10% of the population has reduced expression of UGT1A1 due to a defect in the TATAA box of the UGT1A1 promoter region [29,30]. Based on the findings of this current study, reduced UGT1A1 activity could lead to an increase in PhIP-mediated mutagenicity, because the ability to detoxify PhIP via glucuronidation will be diminished, which could ultimately lead to more mutations and an increase in cancer susceptibility from PhIP exposure. This newly engineered cell line will provide a tool that will afford a more comprehensive study into the mechanisms of PhIP-induced metabolism and mutagenicity as well as other compounds with similar metabolic mechanisms.

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Table 1. Relative quantitation of UGT1A1expression in CHO cells transfected with human UGT1A1 cDNA using the comparative C_T method

Cell Line	UGT1A1 C_T	GAPDH C_T	ΔC_T [a]	$\Delta\Delta C_T$ [b]	UGT1A1 rel. to UV5P3[c]
UV5P3 (control)	38.53	24.75	13.78	0	1.00E+00
5P3hUGT1A1-5	25.24	26.23	-0.99	-14.77	2.79E+04
5P3hUGT1A1-7	25.17	24.71	0.46	-13.32	1.02E+04
5P3hUGT1A1-12	26.4	26.01	0.39	-13.39	1.07E+04
5P3hUGT1A1-14	23.42	26.81	-3.39	-17.17	1.47E+05

[a] ΔC_T = UGT1A1 C_T-GAPDH C_T

[b] $\Delta\Delta C_T = \Delta C_T - \Delta C_{T\ control}$

[c] The relative amount of UGT1A1 = $2^{-\Delta\Delta C_T}$

Table 2. Formation of *N*-hydroxy-PhIP glucuronides from microsomes prepared from CHO cells transfected with human UGT1A1 cDNA and exposed to 25 μM *N*-hydroxy-PhIP[a].

5P3hUGT1A1 Clone no.	*N*-hydroxy-PhIP-N^2-glucuronide	*N*-hydroxy-PhIP-*N*3-glucuronide
5P3hUGT1A1-5	4.3 pmol	nd[b]
5P3hUGT1A1-7	5.3 pmol	nd
5P3hUGT1A1-12	1.3 pmol	nd
5P3hUGT1A1-14	17.0 pmol	1.3 pmol[c]

[a]microsomal protein concentration was 2 mg/ml.

[b]not detected

[c]limit of detection is < 1 pmol

Figure Legends

Figure 1. PhIP dose dependent cell proliferation assay of eleven CHO cell clonal isolates that have been transfected with human UGT1A1 cDNA. Proliferation is reported as the fraction of untreated cells (cells without PhIP).

Figure 2. Ethidium bromide- stained agarose gel of the amplified RT-PCR fragments from CHO cells transfected with UGT1A1. Lane 1, molecular size standard; Lane 2, UV5P3; Lane 3, 5P3hUGT1A1-5; Lane 4, 5P3hUGT1A1-7; Lane 5, 5P3hUGT1A1-12; Lane 6, 5P3hUGT1A1-14.

Figure 3. Formation of *N*-hydroxy-PhIP glucuronides from microsomes (2-5 mg/ml) prepared from 5P3hUGT1A1-14 CHO cells, and exposed to 25 μM *N*-hydroxy-PhIP for 3 h. Open circles are *N*-hydroxy-PhIP-N^2-glucuronide; closed circles are *N*-hydroxy-PhIP-*N*3-glucuronide. Data are the mean of 3 incubations ± SD.

Figure 4. Cell survival and mutations of UV5P3 and 5P3hUGT1A1-14. A) Survival fraction (colony forming ability) after 48 h. B) Mutation frequency at the *aprt* locus. Open circles, UV5P3; closed circles, 5P3hUGT1A1-14. Data are the mean of 6 replicates ± SEM.

Figure 1

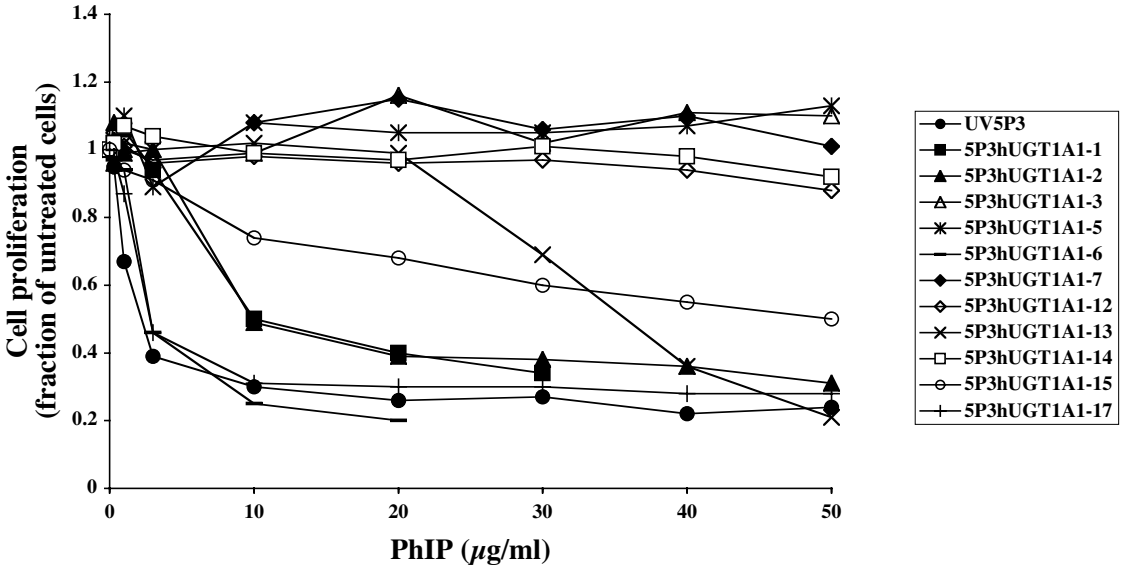

Cell proliferation
(fraction of untreated cells)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
10
20
30
40
50
PhIP (μg/ml)
UV5P3
5P3hUGT1A1-1
5P3hUGT1A1-2
5P3hUGT1A1-3
5P3hUGT1A1-5
5P3hUGT1A1-6
5P3hUGT1A1-7
5P3hUGT1A1-12
5P3hUGT1A1-13
5P3hUGT1A1-14
5P3hUGT1A1-15
5P3hUGT1A1-17

Figure 2

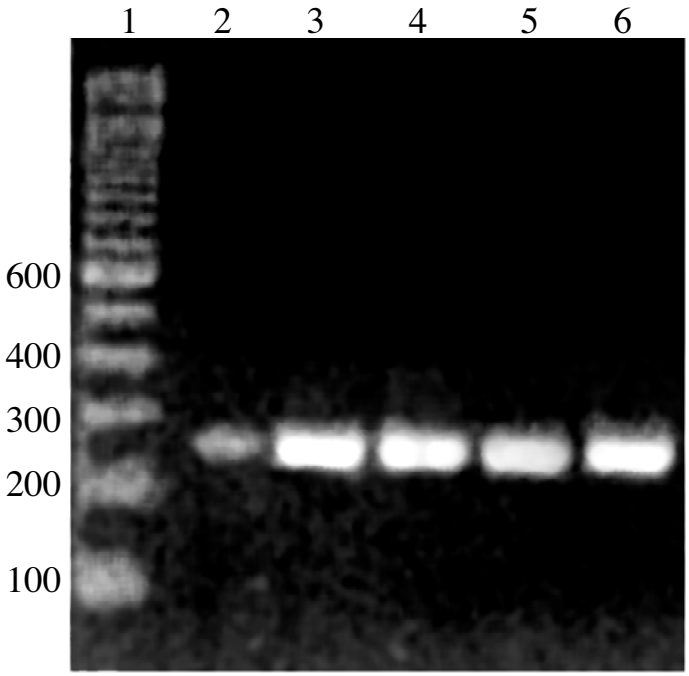


Figure 3

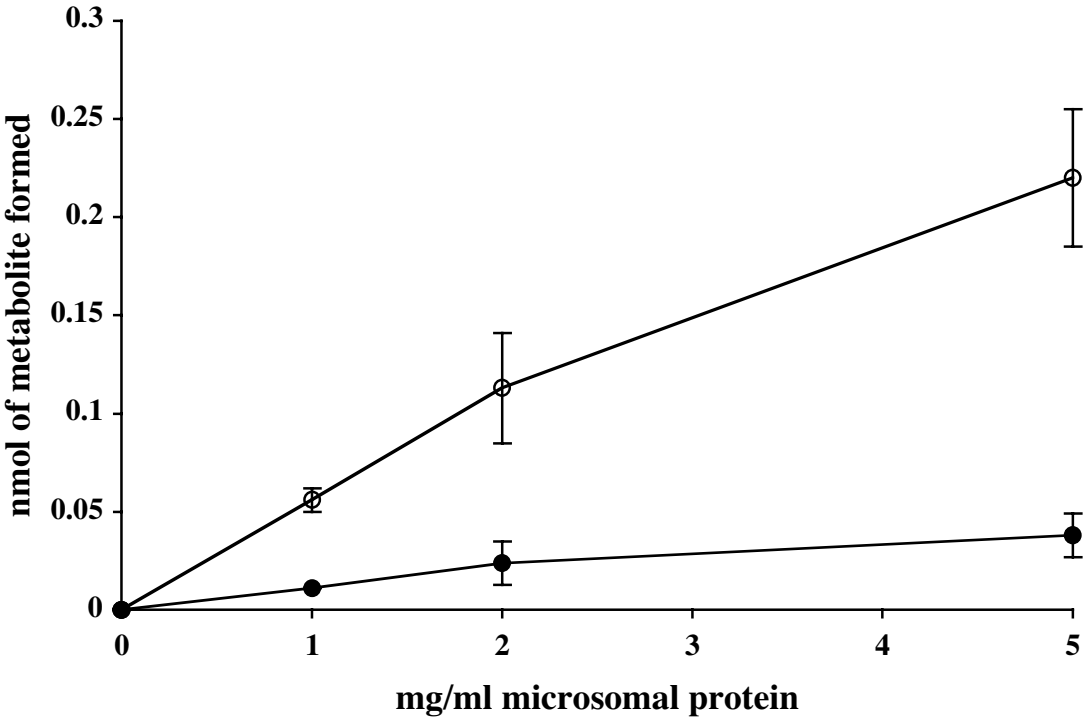
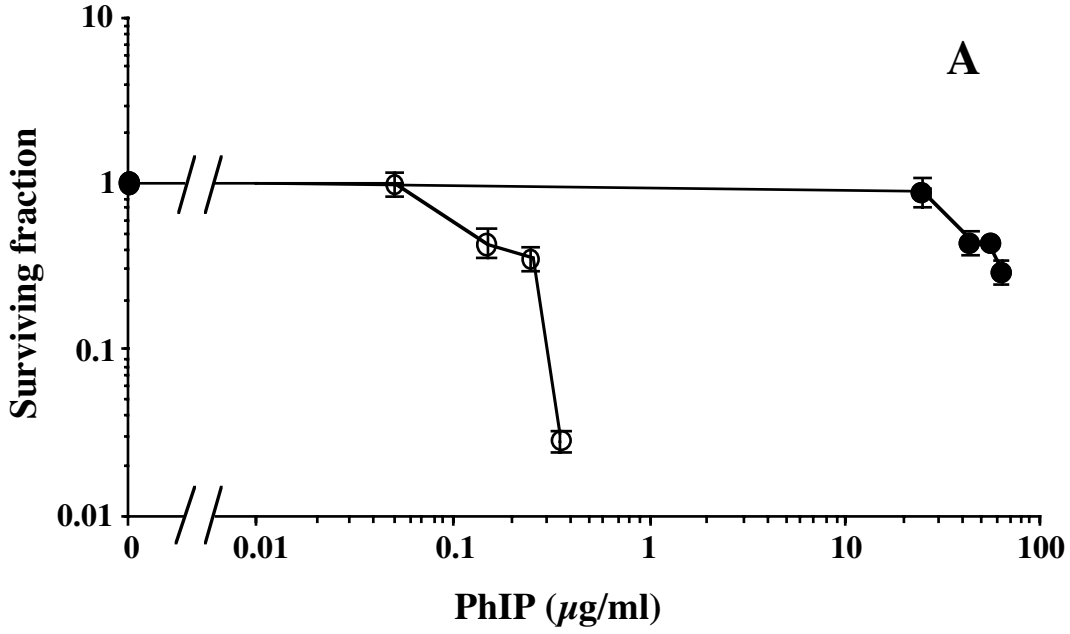
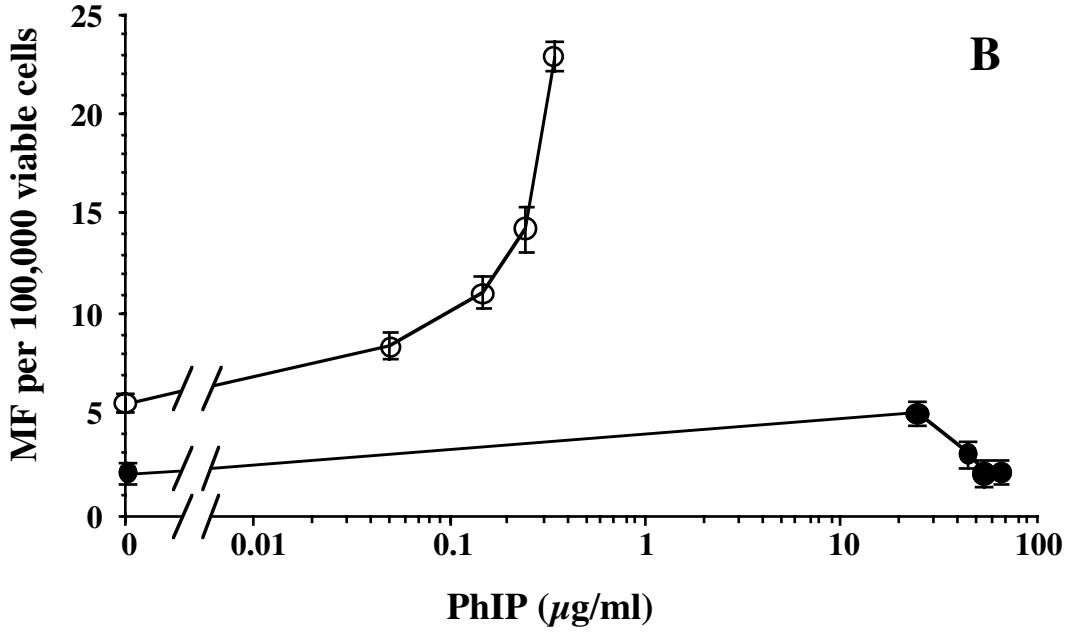

0.3
0.25
0.2
0.15
0.1
0.05
0
nmol of metabolite formed
0
1
2
3
4
5
mg/ml microsomal protein

Figure 4


A
Surviving fraction
10
1
0.1
0.01
0
0.01
0.1
1
10
100
PhIP (μg/ml)


B
MF per 100,000 viable cells
25
20
15
10
5
0
0
0.01
0.1
1
10
100
PhIP (μg/ml)