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4-IPOMEANOL AND 2-AMINOANTHRACENE CYTOTOXICITY IN C3H/10T½ CELLS EXPRESSING RABBIT CYTOCHROME P450 4B1

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Abstract—In the present study, retroviral vectors were used to stably transfer and express the cDNA encoding rabbit CYP4B1 in mouse C3H/10T½ cells. The replication defective retroviral vector was packaged in the ecotropic packaging cell line, GP + E-86, with infectious titer of ~1 × 10^6 cfu/mL. Infection, followed by selection with G418, showed an infection efficiency of approximately 70% for the recipient C3H/10T½ cells. Analysis of ten G418 resistant clones showed that the number of vector inserts ranged from 4 to 13 copies per cell genome. Each clone was positive for microsomal CYP4B1 protein as determined by immunoblotting. Cytochrome P450 4B1 activity was assessed by the cytotoxicity of 4-ipomeanol, a known substrate for P450 4B1 and a model compound for chemical-induced injury to the lung. The initial clonigenic assays showed that 100% toxicity occurred in all the clones after a 96-hr exposure to 250 μM 4-ipomeanol. Parental C3H/10T½ cells were resistant to 4-ipomeanol at concentrations as high as 1 mM. Two clones, designated No. 2 and No. 19, differing in levels of P450 4B1 protein, were characterized further for 4-ipomeanol and other chemical toxicities. A concentration-response study indicated 50% cytotoxicity at 4-ipomeanol concentrations of 1.5 μg/mL for clone No. 2 and 2.5 μg/mL for clone No. 19. A panel of agents representing the aromatic amines, some of which are known or suspected P450 4B1 substrates, were tested for cytotoxicity in clone No. 2. These agents included 2-aminoanthracene, 2-aminonaphthalene, 2-aminofluorene, 2-acetylaminofluorene and 4-aminobiphenyl. Only 2-aminoanthracene gave a clear cytotoxic response reducing the survival fraction of clone No. 2 to 50% at 0.2 μg/mL while affecting parental cells minimally. *In vitro* expression of CYP4B1 provides a new experimental system for further elucidating the cytotoxic and mutagenic effects of P450 4B1 substrates.

Key words: cytochrome P450 4B1; cytotoxicity; aromatic amines; furans

The lung is subject to toxic damage induced by such structurally diverse xenobiotics as the polycyclic aromatic hydrocarbons, aromatic amines, dialkylnitrosamines, indoles and furans [1]. Although there exist some striking species differences in susceptibility to pneumotoxicity from such compounds, one common feature in the susceptible animal species is that biotransformation is a prerequisite for toxicity. The usual result of biotransformation is to facilitate the clearance of xenobiotics from the body. However, the primary step of this process, enzymatic oxidation, can result in the formation of electrophilic intermediates [2]. The tissue concentration of these intermediates is increased by rapid initial oxygenation and the inability of secondary conjugation reactions to keep pace with electrophile production. This situation favors the reaction of the intermediate with nucleophilic centers in cellular proteins and nucleic acids. Adducts with Ca^{2+} regulatory proteins and the ensuing inappropriate activation of Ca^{2+}-sensitive protease and nuclear endonuclease lead to acute cytotoxicity [3]. Adduction to cellular DNA can cause mutation and inhibition of DNA replication, depending on the type and content of the adduct and the fidelity of the DNA repair mechanism [4].

The major enzyme systems known to initiate biotransformation of xenobiotics are the cytochromes P450 [5], flavin-dependent monooxygenases [6], and prostaglandin H synthase [7]. The cytochromes P450 comprise a superfamily of heme-thiolate enzymes that catalyze monooxygenation of a wide array of endogenous and xenobiotic substrates. In mammals, twelve families have been classified to date [8]. Families 1–4 are found mainly in those tissues exposed to xenobiotic chemicals, i.e. liver, intestine, bladder, lung, and skin, and these have broad substrate preference for drugs, agrichemicals, environment pollutants, and organic solvents. The metabolic capability of the cytochromes P450 in xenobiotic metabolism is further extended by the tissue specific differences in isoform composition and the variable induction levels of the individual isoforms.

Studies on species including mouse, rat, rabbit, hamster, and human show certain commonalities of cytochrome P450 isoforms of the lung [9]. All species have isoforms of the 1A, 2B, and 4B subfamilies. Rabbit lung exceeds other tested species in terms of total cytochrome P450 activity, with the three principal isoforms designated 1A1, 2B4 and 4B1. Older names used for these same forms are form 6, form 2 and form 5, respectively (e.g. see Nelson *et al.* [8]). The combined amounts of

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2B4 and 4B1 constitute about 80% of the total cytochromes P450 [10]; cytochrome P450 1A1 (3%) and P450 2E1, 2E2 and 4A4 (female rabbits) account for the remainder [11, 12]. The non-ciliated bronchiolar epithelial (Clara) cell is a prime target for airborne pneumotoxicants and is believed to be the principal cell type in the lung for xenobiotic metabolism [13–15]. Immunolocalization studies in the intact tissue and isolated cell populations show that P450 1A1, 2B4, and 4B1 isoforms are concentrated in the Clara cells of the rabbit lung [10, 16]. The substrate preference of these isoforms (1A1: polycyclic aromatic hydrocarbons [17]; 2B4: dialkylnitrosamines [18]; and 4B1: certain aromatic amines and furans [19]) may explain why these chemicals preferentially attack the Clara cell in rabbits, and perhaps other species as well.

Transgenic cell culture systems expressing individual cytochrome P450 isoforms offer a new method for studying metabolic activation of xenobiotics and the biological effects of their metabolites [20]. The chief advantage of these systems is that the fate of a xenobiotic can be followed from the point of exposure to a final biological end point within a defined cell population. To realize the full benefit of *in vitro* cell systems, the cell should express a single, stable metabolic phenotype to avoid competition for the substrate by multiple cytochrome P450 isoforms and other bioactivation enzymes. In a previous study, we employed a retroviral vector to express the human CYP2A6* in the mouse C3H/10T½ cell [21]. These cells acquired the ability to metabolize the carcinogenic nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and diethylnitrosamines [22] into mutagenic and oncogenic metabolites. In the present work, a similar approach is used to create a furan and aromatic amine metabolic capability in the C3H/10T½ cell by stable expression of the rabbit lung isoform of CYP4B1.

MATERIALS AND METHODS

C3H/10T½ (passage 8) cells were purchased from the ATCC; they were obtained originally from the late Dr. Charles Heidelberger [23]. The cell line GP + E-86 was provided by Dr. Arthur Bank, Department of Genetics and Development, Columbia University, New York, NY. AKR cells were obtained from Ms. Cynthia Innes of the National Institutes of Environmental Health Sciences. Liquid BME, powder DMEM, EMEM, 200 mM glutamine solution, 100 penicillin/streptomycin and FBS were obtained from Life Technologies. Hypoxanthine, xanthine and mycophenolic acid were purchased from the Sigma Chemical Co. 4-Ipomeanol was provided by Dr. Michael Boyd of the National Cancer Institute. 2-AF, 2-AAF, 2-AN, 2-AA, and 4-aminobiphenyl were from Aldrich. The pMV7 vector was provided by Dr. I. B. Weinstein of Columbia University.

* Abbreviations: 2-AA, 2-aminoanthracene; 2-AAF, 2-acetylaminofluorene; 2-AN, 2-aminonaphthalene; 2-AF, 2-aminofluorene; 4-ABP, 4-aminobiphenyl; BME, Basal Medium Eagle; CYP, cytochrome P450; DMEM, Dulbecco's Modified Eagle's Medium; EMEM, Eagle's Minimal Essential Medium; FBS, fetal bovine serum; HBSS, Hanks' Balanced Salt Solition; PAP, 5'-phosphoadenosine-3'-phosphosulfate; RDRV, replication defective retrovirus; and SSC, 0.15 M sodium chloride + 0.015 M sodium citrate.

Growth and maintenance of cell lines

Mouse C3H/10T½ cells (ATCC) were grown and maintained at less than 80% confluency in BME supplemented with glutamine, penicillin-streptomycin and 10% heat-inactivated FBS. The 3T3 fibroblast-derived cell line, GP + E-86 [24], was employed for packaging the retroviral vector. GP + E-86 cells were grown in HXM medium: Dulbecco's Modified Eagle's Medium (DMEM) containing penicillin/streptomycin, 10% heat-inactivated and FBS, hypoxanthine (15 μg/mL), xanthine (250 μg/mL), and mycophenolic acid (25 μg/mL), pH 7.3, for several passages prior to vector transfection. Thereafter, and for virus harvest, GP + E-86 [24] cells were grown in DMEM plus 10% FBS. Mouse AKR cells were used for determining infectious particle titer and were grown and maintained in EMEM supplemented with penicillin/streptomycin, glutamine, and 5% FBS. Frozen stocks of cell lines and clones derived from C3H/10T½ were prepared by freezing 0.5 to 1.0×10^6 cells in 1.0 mL of growth medium plus 10% DMSO for long-term storage in liquid nitrogen.

Vector construction

The cDNA encoding the rabbit CYP4B1 in pBluescript [25] was digested with *Eco*RI to release a 1760 bp fragment that includes 30 bp upstream of the ATG start codon and 210 bp downstream from the TAG stop codon. This digestion eliminates a 200 bp portion of the 3'-untranslated region containing the polyadenylation signal sequences. The retroviral vector, pMV7 (Fig. 1; [26]), was digested with *Eco*RI, dephosphorylated with calf intestinal alkaline phosphatase and the CYP4B1 cDNA inserted by T4 DNA ligase. Vector DNA was amplified by growth in *Escherichia coli* DH5α and the 5' → 3' orientation of the cDNA verified by restriction enzyme mapping.

Vector packaging

The DNA vector containing the CYP4B1 cDNA was packaged into a RDRV by the GP + E-86 cells. Twenty-four hours before transfections, GP + E-86 cells were plated at 5×10^5 cells in 100-mm dishes in DMEM plus 10% FBS. The following day, a calcium phosphate precipitate with 14 μg of vector DNA was prepared in a volume of 400 μL as described by Miller and Rosman [27]. The medium was changed with 6 mL DMEM plus 10% FBS, pH 7.15. The vector:calcium phosphate complex was added dropwise with swirling, and the plates were incubated for 24 hr at which time the cells were about 50% confluent. The medium was removed, and the plates were washed once with 15 mL of fresh medium and incubated with medium containing 400 μg/mL of G418 (Geneticin). The medium was changed at 3-day intervals. After 9 days of selection, no uninfected control cells survived, while the vector-transfected cells were approximately 50% confluent. On day 12 of selection, cells in the 100-mm dishes were confluent and were split to T-75 flasks for expansion to make frozen stocks. For RDRV harvest, transfected GP + E-86 cells were grown in the absence of G418 for two passages in harvest medium (DMEM plus 1 μM hydrocortisone, 0.1 U/mL insulin, 10% FBS) prior to seeding at a density of 5×10^6 cells/T-75 flask. The following day medium was removed from three flasks, replaced with 3 mL of harvest medium, and incubated for 16 hr. The medium was re-

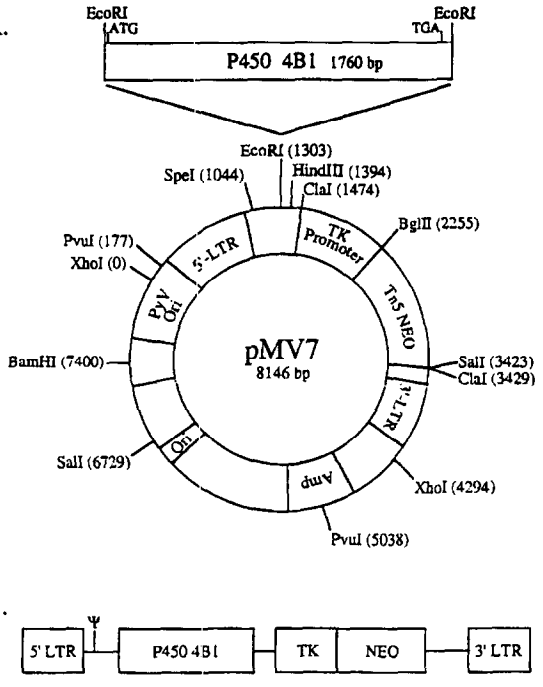

Fig. 1. Structure of the pMV-7 retroviral vector: CYP4B1 construct. The cDNA encoding the rabbit pulmonary CYP4B1 was subcloned into the retroviral vector pMV-7 by standard recombinant DNA techniques outlined under Materials and Methods. Vector was amplified in *E. coli* DH5α and purified twice by CsCl density gradient centrifugation for calcium phosphate transfection into the packaging cell line, GP + E-86, to produce replication defective retroviral particles. Abbreviations: TK, herpes simplex thymidine kinase promoter; neo, neomycin phosphotransferase cDNA; CYP4B1, cytochrome P450 4B1; and LTR, long-terminal repeat of the Moloney murine sarcoma virus.

moved, centrifuged at 1000 *g* for 5 min, and the supernatant used as a source of infectious RDRV. If the supernatant was not used immediately, it was frozen and stored in liquid nitrogen.

Titer of RDRV supernatant

The RDRV supernatant was titered on AKR cells. AKR cells grown in EMEM + 5% FBS were plated at 5 × 10^4 cells/p 35-mm well the day before infection. The RDRV supernatant was diluted serially 10^{-1} to 10^{-6} in BME (-serum) plus polybrene (20 μg/mL) at 4°. Two milliliters of dilution was added to duplicate wells and incubated for 3 hr at 37°. The medium was removed, and fresh medium containing G418 (400 μg/mL) was added. Medium was replaced at 4-day intervals; on day 12 the AKR clones were stained with 1% crystal violet and counted. The supernatant medium from these cells expressed an infectious titer of 1 × 10^6 cfu/mL.

Infection and selection of C3H/10T½ cells

One day prior to treatment with the packaged vector, C3H/10T½ cells were seeded at a density of 2.5 × 10^5 cells/60-mm dish. One milliliter of BME (-serum + 20 μg polybrene) and 1 mL of undiluted infectious medium were added and incubated for 24 hr. The cells were washed twice with HBSS (25°) and trypsinized with 2 mL trypsin-EDTA (Gibco) for 5 min. The trypsinate was diluted 3-fold with BME + serum and centrifuged at 1000 *g* for 5 min; the cell pellet was resuspended in 2 mL BME. The cells were seeded into twenty 100-mm plates at either 100 or 200 cells per plate in 8 mL of BME. G418 selection (400 μg/mL) was started 1 day later. Four plates did not receive G418 and served to determine cloning efficiency of the transfected cells. The medium was changed on day 8. Clones were isolated by ring cloning on day 13.

Genomic DNA isolation and Southern analysis

Genomic DNA was isolated by lysing 5 × 10^6 C3H/10T½ cells in 10 mM Tris (pH 7.5), 1 mM EDTA, 0.5% SDS, and proteinase K followed by phenol-chloroform extraction [28], as described in Sambrook *et al.* [29]. Genomic DNA (4 μg) was digested to completion with *Hind*III. A sample (2 μg) was applied to a 0.7% agarose gel and electrophoresed. DNA from the gel was transferred to Hybond-N by capillary transfer in 20x SSC for 20 hr. The blot was hybridized with a fragment of the neomycin phosphotransferase cDNA with visualization by chemiluminescence using the Renaissance kit (Amersham).

Preparation of microsomes and western analysis of P450 4B1

C3H/10T½ clones were grown to 90% confluency in three T-175 flasks to yield approximately 1.5 × 10^7 cells. Cell layers were washed twice with HBSS and scraped off with 5 mL of buffer consisting of 50 mM sucrose, 50 mM Tris, pH 7.5, 1 mM EDTA, 1 mM benzamidine, 1 mM diisopropylfluorophosphate and 1 mM phenylmethylsulphonyl fluoride. The cells were centrifuged, washed once, and then resuspended in 1.0 mL of scraping buffer with the additional of the protease inhibitors aprotinin, cystatin, leupeptin and E-68, each at 30 μg/mL. Following sonication, the suspension was centrifuged at 6000 *g* for 10 min to obtain a microsomal supernatant that was then centrifuged at 100,000 *g* for 60 min in a Type 50.2 rotor to obtain a microsomal pellet. Microsomal protein was determined by the method of Lowry *et al.* [30], using bovine serum albumin as the standard.

One hundred micrograms of microsomal membrane protein was solubilized in 100 μL of buffer (0.15 M Tris–HCl, pH 6.8, 0.6 M β-mercaptoethanol, 2% SDS, 2% sucrose) by boiling for 5 min. Rabbit lung microsomes served as a positive control for immunoreactive CYP4B1. The solubilized material was applied to 7.5% polyacrylamide-0.1% SDS gels, and electrophoresis was performed at 30 mA/gel. Proteins separated in the gel were transferred electrophoretically to nitrocellulose support. Antibody binding and immunodetection were by the method of Towbin *et al.* [31] and Domin *et al.* [32].

Clonigenic assay for cytotoxicity

Parental and CYP4B1 expressing clones of C3H/10T½ were grown to 80% confluency in T-75 flasks to provide cells for the clonigenic assay. On day 0, cells were reseeded after trypsinization at 200 cells/5 mL/T-25 flask. After 24 hr cells were exposed to chemical agents. Agents were dissolved in acetone and diluted in BME (-serum). Volumes (50–250 μL) of dilutions were added directly to the medium in the T-25 flask such that the final acetone concentration did not exceed 0.1%. The

flasks were capped tightly and incubated for 48 hr. After this period the chemical was removed by a medium change, and the incubation was continued for 9 days to allow colonies to develop. The flasks were stained with 1% crystal violet in 50% methanol, and the colonies were counted.

RESULTS

Selection of CYP4B1 expressing *C3H/10T½ cells*

After infection of growing C3H/10T½ cells, the cultures were reseeded at a cloning density of either 100 or 200 cells per 100-mm dish with G418 selection. Resistant clones began to appear 5 days after selection. Approximately 70% of the cells subjected to the RDRV supernatant were infected and became G418 resistant. Parental cells did not survive G418 treatment. Forty clones were examined microscopically for the next 7–10 days for C3H/10T½-like morphology and for growth rates comparable to parental control clones. Ten colonies with diameters of 5 mm and densely packed cells were chosen for expansion into cell lines. Although some clones were G418 resistant and growing, they were either under the 5-mm diameter or did not show the dense, "cobblestone" packing [33]. Infrequently, clones with lipid-droplet-like inclusions or transformed "swirl" pattern morphology appeared. Clones with these characteristics were not picked. To determine the number of vector inserts in each clone, clones were expanded, and genomic DNA was isolated and digested with *Hind*III. The vector contains a single *Hind*III site between the subcloning site and the neo gene. Digestion results in a collection of DNA fragments that will contain the intact neo region. Hybridization and detection of the neo probe in the genomic DNA are shown in Fig. 2. The vector copy number ranged from 4 to 13. The presence of the neo positive elements in the mouse cell genome indicates that the retroviral mechanism was successful for integration. This finding is an essential prerequisite for establishing stable cell lines in which the new metabolic capability is transmitted and expressed uniformly from cell generation to generation.

Immunoblotting analysis of P450 4B1 protein

Because cytochrome P450 is associated with the membrane fraction of the cell, microsomal membranes were analyzed by immunoblotting to detect the presence and relative level of P450 4B1 in each clone compared with that of rabbit lung microsomal preparations (Fig. 3). The primary antibody [32] reacts to purified rabbit pulmonary P450 4B1 and does not cross-react with the native isoform [34, 35] of parental C3H/10T½ (lane P, Fig. 3) or any protein in the 54,000 molecular weight region. All G418 resistant clones were positive for P450 4B1 and exhibited variable degrees of reactivity with 4B1 antibody. The highest expressing clones, No. 2, 7, and 9, contained approximately one-tenth the P450 4B1 content of native rabbit lung microsomes. Clones No. 8, 10, and 19 showed the lowest level of 4B1 reactivity of the ten clones. Comparing vector copy number to the intensity of the immunoreactive P450 4B1 revealed a basic correlation between number of vector inserts and P450 4B1 content. The lowest expressing clones, 8, 10, and 19, had four to seven inserts, whereas the most intense clones, 2, 7, and 9, had eight to thirteen inserts. The use of the pMV-7 vector-GP + E-86 packaging cell line described here is a highly efficient transgenic method since all of the clones chosen from the G418 resistant cells were positive for P450 4B1 expression.

Activation of 4-ipomeanol

To determine whether the immunoreactive P450 4B1 also possessed enzymatic activity, cytotoxicity to 4-ipomeanol was tested. 4-Ipomeanol [1-(3-furyl)-4-hydroxypentanone] is a naturally occurring furan capable of instigating lethal lung injury [36] following activation by

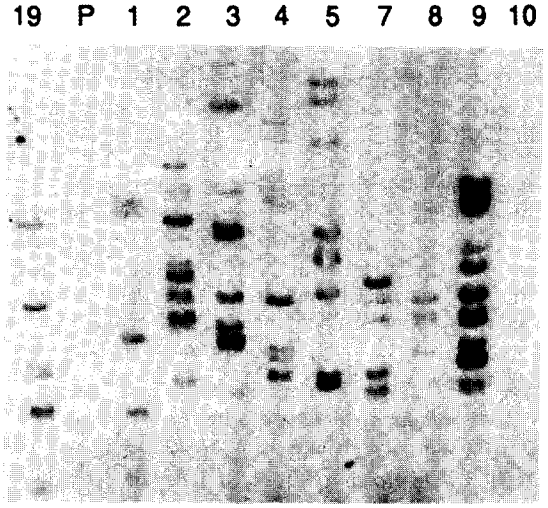

Fig. 2. Southern analysis for neo in the genomic DNA of C3H/10T½ infected cells. C3H/10T½ cells were cloned in the presence of G418 after infection with the retroviral particle. Genomic DNA was isolated and digested with *Hind*III, and capillary blot transfer was performed as described under Materials and Methods. The numbered lanes signify the G418 resistant clones; P, parental C3H/10T½ cells.

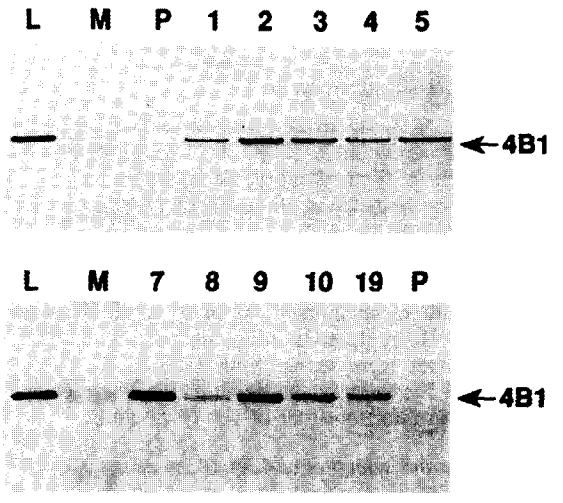

Fig. 3. Western analysis of P450 4B1 protein. Microsomal membrane proteins were separated by 0.1% SDS:7.5% PAGE, and electrophoretically transferred to nitrocellulose sheets as described under Materials and Methods and references therein. Key: L, 9 μg of rabbit lung microsomal protein; M, molecular weight markers; P, 130 μg C3H/10T½ parental cell microsomal protein; numbered clones, 130 μg microsomal protein.

P450 4B1 [37]. Therefore, if P450 4B1 is enzymatically active in the clones, a specific and characteristic cytotoxic response to 4-ipomeanol should be observed. Several clones expressing varying levels of P450 4B1 were incubated with 0.25, 0.50, and 1.0 mM 4-ipomeanol for 96 hr, and the degree of cytotoxicity was assessed by determining the number of cells capable of forming clones after this 48-hr treatment (Table 1). The three concentrations were chosen based on earlier reports comparing the effects of 4-ipomeanol on a series of human lung cancer cell lines [38]. In that study, it was shown that *in vitro* cultures of small cell adenocarcinoma experienced 50% cytotoxicity at ~0.5 mM 4-ipomeanol after a 96-hr exposure. In our experiments, the survival fraction of all P450 4B1 expressing clones, independent of the intensity of immunoreactive P450 4B1, was zero at the lowest (0.25 mM) of the 4-ipomeanol concentrations.

Clones No. 2 and 19, which represent the high and low extremes of immunoreactive P450 4B1, were characterized further for a 4-ipomeanol cytotoxic response over the concentration range of 0.1 to 10 μg/mL (0.6 to 60 μM) (Fig. 4). Clone No. 2 exhibited 50% toxicity at a 4-ipomeanol concentration of 1.5 μg/mL. The response curve for clone No. 19 was shifted so that 50% cytotoxicity was achieved at 2.5 μg 4-ipomeanol/mL. The complete resistance of parental C3H/10T½ cells and the increased sensitivity to 4-ipomeanol toxicity in clone No. 2 compared with clone No. 19 coincides with the relative level of P450 4B1 in the cells.

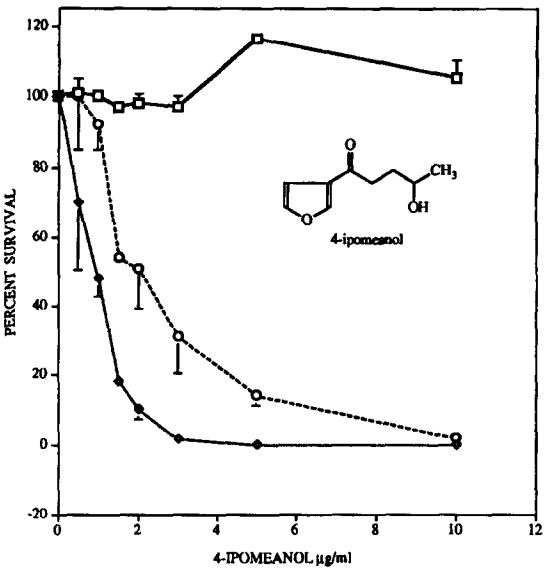

Fig. 4. Concentration dependence of 4-ipomeanol cytotoxicity. Parental, clone No. 2 and clone No. 19 cells were seeded at an initial density of 200 cells per T-25 flask in 5 mL of BME + 10% FBS. After 24 hr, 4-ipomeanol was added at the indicated concentrations for a 48-hr exposure period after which time the medium was changed. Clones were stained and scored 9 days later. Key: parental (□—□); clone No. 2 (◇··◇); and clone No. 19 (○-○).

Activation of furans and aromatic amines

After the demonstration of 4-ipomeanol selective toxicity, a group of chemical agents known to have *in vivo* toxic and carcinogenic effects was tested for cytotoxicity in parental and clone No. 2 cells (Fig. 5). Rabbit P450 4B1 is capable of producing mutagenic products from 2-AF, 2-AAF and 2-AA [39]; however, the cytotoxicity to their metabolites in mammalian cells has not been determined. In the present study, 2-AA proved highly cytotoxic, reducing cell survival by 50% at 0.2 μg/mL (1×10^{-6} M) and killing all cells at 1 μg/mL. Parental cells showed only marginal toxicity of (approximately 14%) at the highest (10 μg/mL) exposure level. 2-AN, 4-ABP, 2-AF and 2-AAF exerted no cytotoxic effect on either parental or clone No. 2 cells.

Table 1. 4-Ipomeanol cytotoxicity in selected CYP4B1 expressing C3H/10T½ clones

Clone No.	Vector copy number	Cloning efficiency (%)	4-IPO Cytotoxicity: Cloning efficiency (%)
Parental	0	20.7 ± 1.7	18.9 ± 1.80
2	9	27.1 ± 1.9	0
3	8	13.4 ± 3.2	0
4	7	19.3 ± 0.6	0
5	8	19.2 ± 2.9	0
8	4	17.4 ± 1.6	0
9	12	8.5 ± 2.3	0
10	4	13.0 ± 1.0	0
19	7	25.1 ± 2.8	0

The clonigenic assay was established for the designated clones as described under Materials and Methods. Cells were treated for 96 hr with either 0 (control), 0.25, 0.50 or 1.0 mM 4-ipomeanol. Numbers are reported for the 0.25 mM concentration; results for clone No. 2 were obtained at 0.10 mM concentration. The cloning efficiency for parental cells was 20.7% in the absence of 4-ipomeanol and 18.8, 16.1 and 17.4% in the presence of 0.25, 0.50 and 1.0 mM 4-ipomeanol, respectively. Results are the means ± SEM of five determinations in a single trial.

DISCUSSION

The present study describes the construction and characterization of a derivative of the C3H/10T½ cell that expresses a specific metabolic capability for chemicals toxic to the lungs of certain species. The specificity in the cell line is determined by overexpression of isoform CYP4B1. Rabbit P450 4B1 has been characterized thoroughly in both isolated lung microsomes and reconstituted systems for its substrate preference and ability to form mutagenic products from these substrates. The choice of the aromatic amine compounds reported here was based on this earlier work. The first indication that the C3H/10T½:CYP4B1 clones had a new metabolic phenotype came from the screening and concentration-response results with 4-ipomeanol. This compound is a model toxin for studying selective toxicity in the lung [40]. Boyd *et al.* [36] and Boyd and Burka [41] showed that *in vivo* pneumotoxicity of 4-ipomeanol could be blocked by inhibitors of cytochrome P450. Devereux *et al.* [42] extended this work by fractionating rabbit lung into Type II, non-ciliated bronchiolar epithelial (Clara Cell) alveolar macrophage and mixed cell populations

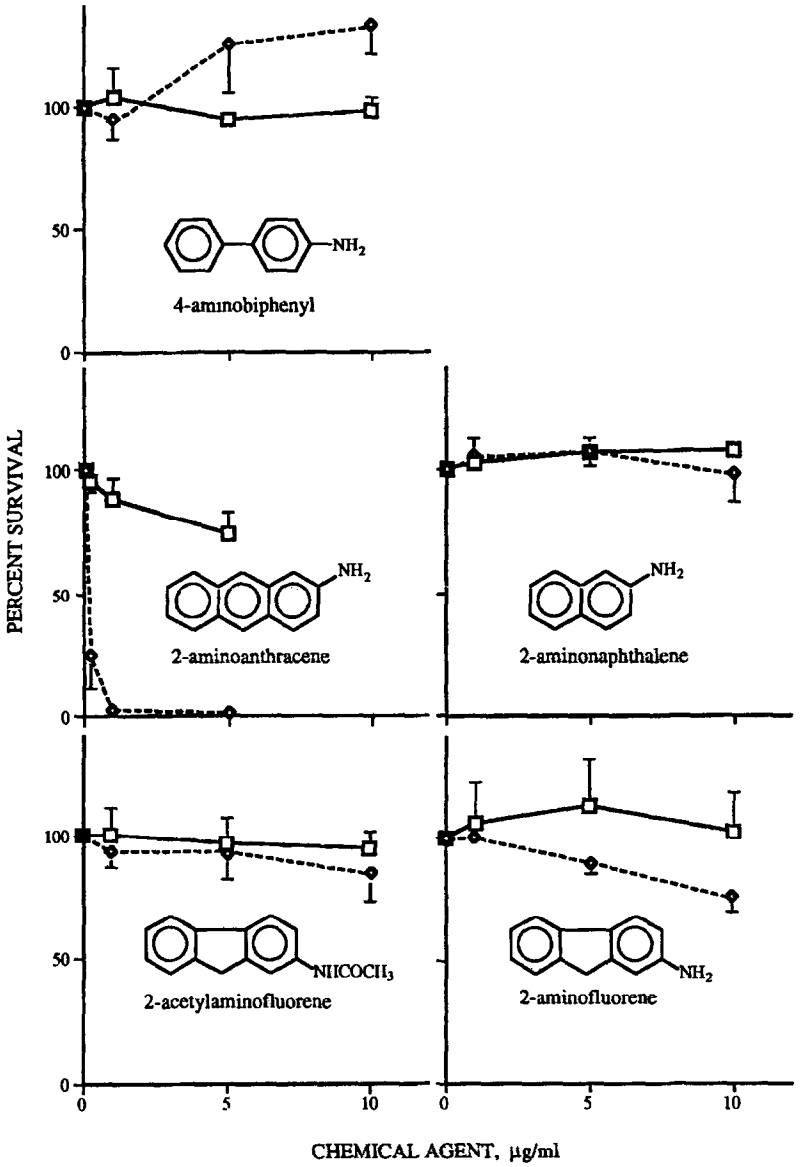

Fig. 5. Cytotoxicity of selected aromatic amines and chemical agents. The clonigenic assay was established as described in Materials and Methods. Agents were added to the T-25 flasks at the indicated concentrations (0–10 μg/mL) for a 48-hr exposure time. Results are the means ± SEM of three separate trials with three determinations per data point. Key: parental (□—□); and clone No. 2 (◇ ◇).

and showed that the metabolic activation of [^{3}H]4-ipomeanol was highly localized in the Clara cell consistent with the early destruction of these cells in *in vivo* studies. Slaughter *et al.* [43] and Wolf *et al.* [37] characterized 4-ipomeanol metabolism by P450 2B4 and 4B1 in rabbit lung microsomes and in reconstituted systems. The basic finding was that P450 4B1 and 2B4 were the major catalytic agents for the conversion of 4-ipomeanol into intermediates capable of forming protein adducts. Purified P450 4B1 produced these adducts 10-fold greater than 2B4. Czerwinski *et al.* [19] expressed fourteen different human, rodent, and rabbit P450 isozymes in the HepG2 cell using the vaccinia virus system. Incubation of cells with [^{3}H]4-ipomeanol resulted in DNA adduct formation in cells expressing human CYP1A2, 3A3, 3A4, 2F1, and 4B1 and rabbit CYP4B1. Rabbit P450 4B1 exceeded all isoforms for 4-ipomeanol adduct formation being 10-fold greater than that formed in cells expressing human CYP1A2. The behavior of our CYP4B1 clones parallels the above findings; the higher the expression level of CYP4B1, the more sensitive the cell is to cytotoxicity by 4-ipomeanol. In the absence of CYP4B1 (parental C3H/10T½ cells), no toxicity was observed at any of the 4-ipomeanol concentrations tested. This result served to confirm that the CYP4B1 expressed by the clones is sufficiently active to produce levels of metabolites capable of causing cytotoxicity.

Expression of CYP4B1 also conferred a new metabolic phenotype in the C3H/10T½ cell for toxicity to 2-AA. Other promutagenic aromatic amines, 2-AN, 2-AF, and 2-AAF had little or no effect on cell survival. Common to these chemicals is hydroxylation of the

amino group and several ring carbons by cytochrome P450 [44]. The metabolism of 2-AF and 2-AAF to DNA-binding agents involves several different pathways beginning from the N-hydroxy intermediate. The N-OH derivative of 2-AF forms DNA adducts either directly in an acid-catalyzed reaction or by a PAP-dependent sulfonylation to create the electrophilic N-sulfonyloxy ester of 2-AF [45]. For 2-AAF, N-hydroxylation is followed by deacetylation to N-OH-AF, which can then follow the acid catalyzed and/or N-sulfonyl ester pathway to ultimately yield 2-AF adducts with guanine [46]. Sulfonylation of 2-AAF results in an electrophile that forms guanine:acetylaminofluorene adducts. An alternative to sulfonylation is acetylation to the N-acetoxy intermediate. 2-AAF, after deacetylation and N-hydroxylation, and N-OH 2-AF can be converted directly to the N-acetoxy electrophile by transfer of an acetyl group from acetyl-CoA by the enzyme *N*-acetyltransferase [44]. "Rapid acetylator" phenotypes form higher levels of DNA:AF adducts and mutagenic products than those of control animals with the normal level of O-acetylation activity and acetyltransferase enzyme content [47].

Aune *et al.* [48] showed that P450 4B1 in rabbit lung microsomes is capable of N-hydroxylation and C-oxidation of ring carbons 1, 3, 5, 7, and 9 of the aminofluorenes. Metabolism is accompanied by production of agents that induce Salmonella mutagenesis, an effect that can be blocked by antibodies to P450 4B1. Paroxon, a deacetylase inhibitor, blocked formation of mutagens from 2-AAF but not 2-AF, indicating that activation of 2-AAF proceeds by an initial deacetylation to 2-AF followed by N-hydroxylation by P450 4B1. Rabbit P450 4B1 also catalyzes, to the same extent as human liver CYP1A2, the formation of 2-AF:DNA adducts in accord with these mutagenicity results [19]. Since this same P450 4B1 is active in our clones, the N-hydroxylating activities for the aromatic amines should be operating. The C3H/10T½ cell has the enzymatic capacity for sulfonylation and deacetylation [49, 50] but the activities may not be sufficient to generate concentrations of reactive intermediates needed to induce toxicity. Additionally, since this cell line is capable of glucuronidation [49], this inactivation pathway may remove reactive intermediates before they can exert their cytotoxic effects. Although DNA adduct formation is believed to be an initial event in mutation and chemical carcinogenesis, it is not clear whether the intermediates in the adduction pathway are the same as those that instigate cytotoxicity. It is of interest that Hansen *et al.* [51] have shown that a clone of C3H/10T½ expressing the human CYP2B1 also expressed a cytotoxic response to 2-AAF (50% survival, 10 μg/mL 2-AAF). As in the present study, parental cells were completely resistant to killing by this aromatic amine. When 2-AAF metabolites were characterized in this clone, it was found that the levels of ring-hydroxylated intermediates were increased, particularly the 1, 3, and 5/9 carbon derivatives. Thus, it is possible that a pathway involving C-hydroxylation is dominant in cytotoxicity for the C3H/10T½ cell. The determination of the types and concentration of aromatic amine metabolites and whether DNA binding occurs apart from cytotoxicity will be necessary to differentiate the toxic from the adduction pathway in the CYP4B1 expressing clones.

Conversion of 2-AA and 2-AN to mutagenic intermediates can be initiated by hydroxylation at C-1 as well as at the amino group [44]. Detailed studies on 2-AN have shown that C-hydroxylation leads to a reactive naphthoquinone that can form adducts with adenine and guanine bases in DNA. In this pathway, the naphthol from P450 hydroxylation is oxidized to the quinone and does not utilize the secondary enzymes required for 2-AF and 2-AAF metabolic activation. Like 2-AF and 2-AAF, the N-hydroxy derivative of 2-AA and 2-AN can form DNA adducts by the acid-catalyzed and sulfuric acid ester routes [44]. A likely explanation for the complete lack of 2-AN cytotoxicity is that P450 4B1 does not bind 2-AN and thus does not effect either C- or N-hydroxylation. Identifying the metabolites from radiolabeled 2-AA and 2-AN produced by the clones is necessary to confirm this substrate preference mechanism to explain the differing effects of 2-AA and 2-AN. Such a study would also reveal whether P450 4B1 has a positional specificity for C-1 over N-hydroxylation for 2-AA. The latter result could be helpful in defining which pathway, C-hydroxylation to the quinone or N-hydroxylation to the sulfuric acid ester, predominates in 2-AA cytotoxicity.

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