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INDUCTION OF SISTER CHROMATID EXCHANGES IN HUMAN AND RAT HEPATOMA CELL LINES BY CYCLOPHOSPHAMIDE AND PHOSPHORAMIDE MUSTARD AND THE EFFECTS OF CYTOCHROME P-450 INHIBITORS

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Abstract—The activity of the cytochrome P-450-associated metabolic pathway in human (HepG2) and rat (H4-II-E) hepatoma cells was examined The genotoxic activities of cyclophosphamide and its direct acting metabolite, phosphoramide mustard, were studied in the hepatoma cells as cyclophosphamide is known to be metabolized by phenobarbital-inducible cytochrome P-450-associated metabolic activity HepG2 and H4-II-E demonstrated the capacity to activate cyclophosphamide to forms capable of inducing sister chromatid exchanges in a concentration-dependent fashion Phosphoramide mustard induced a similar pattern of sister chromatid exchanges at concentrations three orders of magnitude lower than cyclophosphamide The cytochrome P-450-associated enzyme inhibitors, SKF-525A and metyrapone, were found to reduce the level of cyclophosphamide-induced sister chromatid exchanges in HepG2 and H4-II-E, suggesting that cyclophosphamide was activated by this pathway in both hepatoma lines Direct evidence for the presence of mRNA transcript coding for a phenobarbital-inducible cytochrome P-450 was demonstrated in HepG2 cells by Northern blot analysis Comparison of genotoxic responses in human and rat hepatoma cells may allow for an evaluation of responses by different species to potentially mutagenic chemicals

The majority of *in vitro* mutagenicity assays rely on exogenous activation systems such as S9 mixes derived from rodent liver homogenates Aside from technical difficulties inherent in the preparation and use of S9, the metabolites generated by such systems may differ from those generated by intact cells [1] An alternative to subcellular activating systems is the use of continuous cell lines with endogenous activating capacity which can also act as target cells for documenting genotoxic responses Two cell lines which may be useful in this regard are the HepG2 and H4-II-E (H4‖) cell lines. HepG2, an epithelial cell line derived from a human hepatoblastoma [2], has been shown to endogenously metabolize cyclophosphamide, benzo[*a*]pyrene, 7,12-dimethylbenzo-[*a*]anthracene, diethylstilbestrol, aflatoxin B_1 [3–8] and benzidine.¶ HepG2 has also been shown to be amenable for use in cytogenetic analyses [3] H4, an epithelial cell line derived from a rat hepatoma [9], has also been shown to metabolize a variety of promutagens to genotoxic products and to be useful in both cytogenetic and gene mutation assays [3, 4, 10]

In addition to determining whether a test chemical may be metabolized to genotoxic products, HepG2 and H4 cells may be useful in elucidating the pathway(s) by which mutagenic chemicals are activated In the present study, we demonstrate through the use of specific cytochrome P-450-associated enzyme inhibitors, SKF-525A and metyrapone [11, 12], that cyclophosphamide is activated by this pathway in both hepatoma cell lines. This is the same pathway by which this drug has been shown to be activated to its alkylating metabolite phosphoramide mustard *in vitro* and *in vivo* [13–15].

Since a majority of chemical carcinogens require metabolism of the parent compound to chemical species that interact with genetic material [16], it would be useful to have cellular systems to determine whether a chemical requires activation and by what metabolic processes or pathways it is activated Also, since many tests, including the long-term bioassay for carcinogenicity, utilize rodents as models, it is important to compare the responses between rodents

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‖ Abbreviations H4, H4-II-E rat hepatoma cells, CY, cyclophosphamide, PM, phosphoramide mustard, R I , replication index, SCE, sister chromatid exchange(s), BrdUrd, 5-bromo-2′-deoxyuridine, 1 X SSC, standard saline citrate, 0 15 M NaCl/0 015 M trisodium citrate, 1 X PM, 0 02% ficoll 400, 0 02% bovine serum albumin, 0 02% polyvinylpyrrolidone-360, 1 X E buffer, 0 01 M sodium phosphate buffer, pH 7 4, kb, kilobase pair, bp, base pairs, and SDS, sodium dodecyl sulfate

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and human cells HepG2 and H4 cell lines may be useful in the development of such assays

MATERIALS AND METHODS

Cell lines The four cultured cell lines used in these studies were. HepG2, derived from a human hepatoblastoma; H4-II-E (H4), derived from a rat hepatoma; V79, a subclone of a Chinese hamster lung cell line, and IMR-90, a diploid, human embryonic lung fibroblast. All cells and their growth conditions have been described previously [3]

Pretreatment with inhibitors. For experiments dealing with SKF-525A (provided by Drs. Katherine Kennedy and Paul Mazel, Department of Pharmacology, George Washington University Medical Center) and metyrapone (Sigma, St. Louis, MO), cell cultures were incubated in the presence of the indicated concentrations of the inhibitor for 15 min before initiation of SCE studies. SKF-525A and metyrapone were dissolved in serum-free minimum essential medium (MEM, GIBCO, Grand Island, NY) and added immediately to the cell cultures.

SCE studies For SCE analysis, 2–5 10^5 cells were plated into 25 cm^2 tissue culture flasks (Corning) and allowed to incubate overnight. Cyclophosphamide (CY; Cytoxan, Mead Johnson, Evansville, IN) or phosphoramide mustard (PM; prepared by Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD) and 10 μg/ml of 5-bromo-2'-deoxyuridine (32 μM; BrdUrd; Sigma) were added simultaneously and left in the medium of the growing cells for two cell cycles. Two protocols were used for inhibitor studies In the first protocol, hepatoma cultures (HepG2 and H4) were exposed to CY and/or SKF-525A in serum-free medium for 30 or 60 min after the pretreatment period The medium was then removed, and the cells were rinsed with phosphate-buffered saline (PBS, pH 7 4). Fresh medium with 10 μg BrdUrd/ml and the normal complement of serum was added In the second protocol, the inhibitor was added at the same time as CY and BrdUrd and left in contact with the cells for the entire culture period All cultures were allowed to grow in the dark for the two cell cycles (HepG2· 72 hr; H4. 32–36 hr; IMR-90 48 hr, V79 24 hr) Harvesting of cultures, preparation of slides, and analysis of SCE and replication index (R I) have been described [3]. The R.I is an indication of the cell replication kinetics where a value of 2 0 indicates the population went through an average of two cell cycles during BrdUrd exposure Values less than 2 0 indicate a lengthening of the cell cycle interval and values greater than 2 0 indicate a shortening of the interval.

Statistical analyses SCE frequency values were subjected to square root transformation to equalize the variances [17]. The statistical significance of differences between comparison groups was determined by Student's *t*-test All values represent the

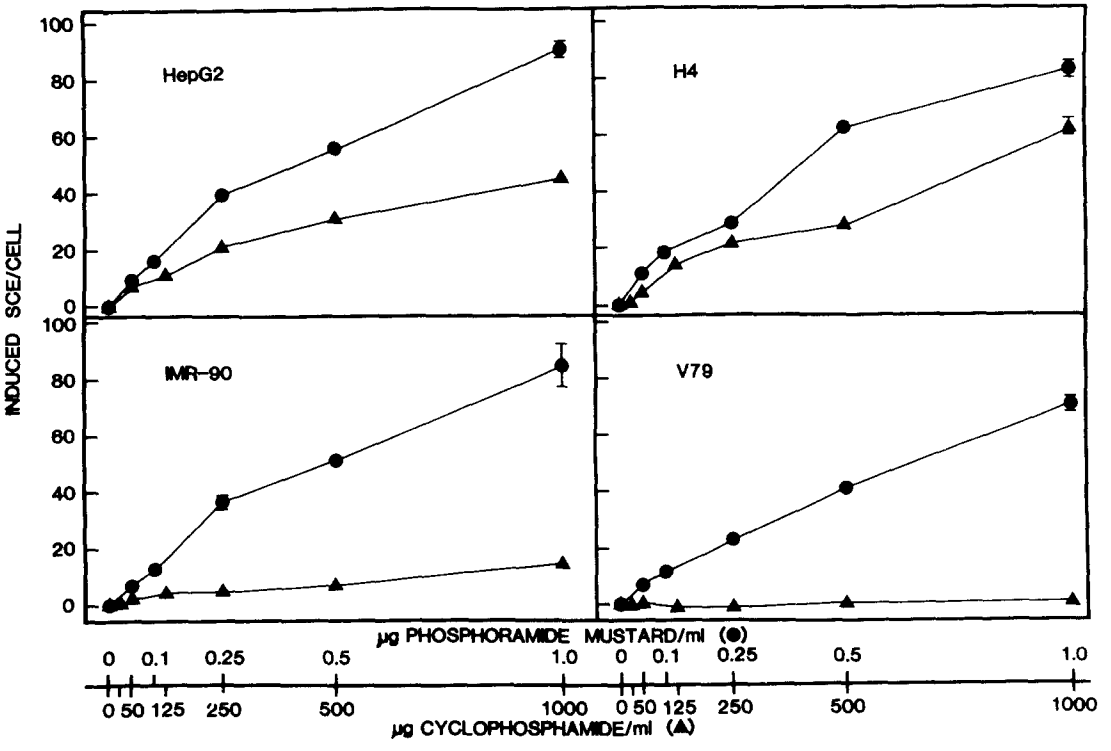

Fig 1 Comparison of induced SCE/cell frequencies in the four cultured mammalian cell lines, HepG2 H4, IMR-90 and V79, after exposure to cyclophosphamide (▲) and phosphoramide mustard (●) Cells were exposed to cyclophosphamide or phosphoramide mustard for their entire culture period (i e two cell cycles) Background SCE levels were subtracted from each point Error bars indicate the standard error of the mean (S E) while points with no bar indicate that the S E falls within the symbols

combined results from at least two independent experiments.

RNA isolation and Northern blot analysis. RNA was isolated from HepG2 cells and from a normal human liver sample obtained from a kidney transplant donor who was maintained on life support systems until kidney removal and lobectomy. The normal liver sample was utilized as a positive control for P-450 expression and was supplied by D. Davies and A. Boobis, Department of Clinical Pharmacology, Royal Postgraduate Medical School, London, England. In addition, RNA was isolated from HL60 cells (a human promyelocytic cell line) and NIH 3T3 cells (a mouse fibroblast cell line) which were utilized as negative controls for P-450 expression.

Approximately 6×10^8 log phase cells or 2 g of tissue were used for each isolate. RNA was isolated with guanidine thiocyanate by the method of Schweizer and Goerttler [18] and enriched for poly(A)RNA by two cycles of oligo(dT)-cellulose chromatography [19]. Electrophoresis of poly(A)RNA samples on horizontal denaturing formaldehyde agarose gels with subsequent transfer to nitrocellulose membranes was performed as previously described [20]. Membranes were hybridized to a [^{32}P] nick-translated P-450 probe at a specific activity $>5 \times 10^8$ cpm/μg DNA for 17 hr at 42° in 50% deionized formamide, 5 × SSC, 10 × PM, 5 × E buffer and 0.1% SDS with 0.5 mg/ml yeast tRNA carrier. The P-450 probe used was R17, a PstI/PstI 1100 bp cDNA P450 probe supplied by M. Adesnik and M. Atchison, New York University [21]

Membranes were washed at high stringency (2 × SSC, 0 1% SDS at room temperature for 30 min with a total of three changes, and 0.1 × SSC, 0.1% SDS at 50° for 30 min with a total of two changes), air dried, and then exposed to Kodak XR-5 X-ray film with intensifying screens.

RESULTS

The induction of SCE in the four cell lines by CY and PM is shown in Fig 1. The concentration responses produced by CY in the two hepatoma lines (H4 and HepG2) and the absence of genotoxic activity in the lung fibroblasts (V79 and IMR-90) were similar to studies reported previously [3]. These data support the previous observations that the hepatoma lines can metabolize CY to mutagenic forms while the lung fibroblasts do not. Since PM is the direct acting genotoxic metabolite of CY [15], the activity of PM in the four cell lines was examined. PM induced similar levels of SCE in all four cell lines with similar concentration–response patterns. The induced SCE/cell ± S E. for each cell line at 1.0 μg PM/ml were: HepG2, 90.1 ± 2.9, H4, 82.9 ± 3.0, V79, 70.2 ± 2.7; and IMR-90, 85 4 ± 7.3. It is noteworthy that the concentrations of PM which induced similar levels of SCE as Cy in the hepatoma cells were over three orders of magnitude less than those for CY. There was a decrease in R.I. values (Table 1) and few mitotic figures were found at PM concentrations above 1.0 μg/ml, suggesting high levels of cytotoxicity

Phenobarbital, an inducer of cytochrome P-450-associated enzyme systems responsible for the bioactivation of CY [22], was reported earlier to exert no detectable alteration on CY-induced SCE in the HepG2 and H4 hepatoma lines [3] Therefore, the agents SKF-525A and metyrapone were examined for their reported capacity to inhibit the activity of these cytochrome P-450-associated enzyme systems [11] and possibly alter SCE induction by CY in the hepatoma cells

Two protocols were utilized to determine whether SKF-525A, a competitive inhibitor of the substrate site of cytochrome P-450-associated enzymes [23], could reduce the level of SCE induced by CY Cells were exposed simultaneously to SKF-525A and CY

Table 1 Replication index (R I) values for the four cultured cell lines after incubation with increasing concentrations of cyclophosphamide and phosphoramide mustard

	Concn (μg/ml)	R I values*			
		V79	H4	IMR-90	HepG2
Cyclophosphamide	0	1 7	2 5	2 0	2 2
	25	1 6	2.5	2 0	
	50	1 6	2 3	1 9	2 2
	125	1 6	2 3	1 8	2 1
	250	1 6	2 4	1 8	1 9
	500	1 5	2 1	1 9	2 0
	1000	1 5	2 0	1 7	1 8
Phosphoramide mustard	0	2 0	2 0	1 9	2 2
	0 05	2 0	2 0	1 9	2 3
	0 1	2 0	2.1	1 9	2 0
	0 25	1 9	2 0	2 0	2 0
	0 5	1 9	2 0	1 8	2 0
	1 0	1 9	1 9	1 6	1 9
	5 0	1 3	1 0		1 0
	10 0	1 0			

Cultures were grown for two cell cycles in the presence of 5-bromo-2'-deoxyuridine

* R I × 100 = 1 (% of metaphase cells in M_1) + 2 (% of metaphase cells in M_2) + 3 (% of metaphase cells in M_3 and higher)

Table 2 Effect of short-term SKF-525A exposures on CY-induced SCE in H4 and HepG2 hepatoma cells

Cell line	Time (min)	Induced SCE/cell: CY (500 μg/ml)	SKF-525A 10^{-4} M	SKF-525A 10^{-5} M	SKF-525A + CY (500 μg/ml) 10^{-4} M	SKF-525A + CY (500 μg/ml) 10^{-5} M
H4	30	4 98 ± 0 65	−0 75 ± 0 67	1 60 ± 0 79	−0 20 ± 0 84	2 93 ± 0 38
H4	60	6 58 ± 0 65	0 17 ± 0 89	1 63 ± 1 02	0 88 ± 0 86	2 73 ± 0 83
HepG2	60	6 38 ± 1 61		−0 14 ± 0 97		2 12 ± 0 84

Cell cultures were pretreated with the indicated concentrations of SKF-525A for 15 min Cultures were then exposed to SKF-525A with or without cyclophosphamide (500 μg/ml) for 30 or 60 min The chemicals were removed and replaced with growth medium for the entire culture period (two cell cycles) Background SCE levels were subtracted from each point Values are listed as means ± standard error of the mean

for 30 or 60 min in the first protocol. In the second protocol, SKF-525A and CY were left in contact with cells for the entire culture period.

SKF-525A alone, at the concentrations utilized in both protocols, had no effect on baseline SCE values (Table 2 and Fig 2) When combined with CY using the first exposure scheme, the inhibitor was effective in suppressing induced levels of SCE (Table 2) For the H4 cell line at 10^{-5} M SKF-525A, there was a 41 and a 59% reduction in CY-induced SCE at 30 and 60 min respectively. The 10^{-4} M concentration essentially abolished all CY-induced SCE at both time points A 67% reduction of SCE with 10^{-5} M SKF-525A was seen in HepG2 cells after a 60-min incubation with CY These results indicate that the hepatoma cells activate CY to SCE-inducing form(s) by a pathway that can be inhibited by SKF-525A, presumably the cytochrome P-450-associated enzymatic pathway

When hepatoma cells were exposed to CY and SKF-525A for the entire culture period, there was no discernible effect on SCE induction by CY in either cell line (Fig 2) Exposure to higher concentrations for the extended incubation times proved cytotoxic, and no mitotic figures were found for scoring SCE.

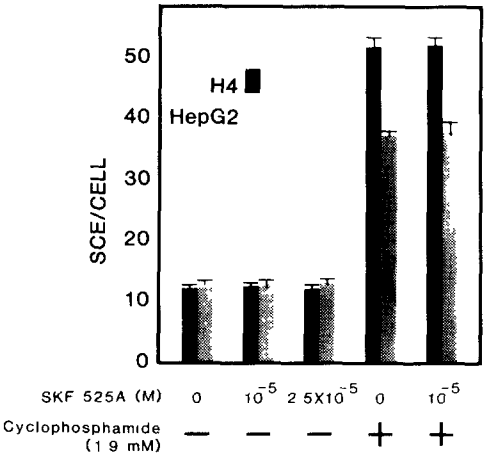

Fig 2 Effect of SKF-525A on CY-induced SCE in HepG2 and H4 hepatoma cells Cells were pretreated with the indicated concentrations of SKF-525A for 15 min Cultures were then exposed to SKF-525A with or without cyclophosphamide (500 μg/ml, 1 9 mM) for the entire culture period (two cell cycles) Error bars indicate the standard error of the mean No significant differences between control cultures and SKF-525A containing cultures were seen

Metyrapone was tested for inhibitory activity by exposing the hepatoma cultures to metyrapone and CY for the entire culture period (Fig. 3) Cultures with metyrapone alone (10^{-5}–10^{-3} M) showed SCE levels that were not significantly different from baseline. Metyrapone decreased the CY-induced SCE in a concentration-dependent manner (e g CY-induced SCE/cell ± S.E decreased from 37 0 ± 0 7 to 27 2 ± 0 9 and 45 4 ± 1 3 to 32 2 ± 1 5 at 10^{-3} M metyrapone for HepG2 and H4 respectively) These decreases were significant at all concentrations of metyrapone ($P \leq 0.001$ at all concentrations)

Studies involving HepG2 metabolic activation of CY strongly suggest the presence of the cytochrome P-450-dependent microsomal monooxygenase system in these cells To directly determine if the cytochrome P-450-associated gene family is transcriptionally active in HepG2 cells, cytochrome P-450 expression was analyzed by hybridizing Northern blots with a recombinant plasmid containing a 1 1 kb cDNA clone of a rat P-450 gene Ethidium bromide staining properties of the denaturing agarose gels before and after nitrocellulose transfer confirmed that equal amounts of RNA were electrophoresed on each lane with subsequent complete transfer to the nitrocellulose membrane filters Figure 4 indicates that human liver (lane 1) and HepG2 cells (lane 2) contain P-450 specific transcripts of approximately 3.3 kb No P-450 specific transcripts were detected in HL60 cells (lanes 3 and 4) or NIH 3T3 cells (lane 5) which represented negative control RNA preparations This blot was washed (boiled for 4 min in distilled water) and rehybridized with an actin probe which demonstrated that all lanes had approximately equal amounts of hybridizable RNA

DISCUSSION

The present study provides both direct and indirect evidence that the human hepatoma cell line HepG2, and the rat hepatoma cell line, H4, contain an active cytochrome P-450-dependent monooxygenase system capable of metabolizing CY to a genotoxic form(s) Concentration–response studies indicate that the two hepatoma cell cycles had similar levels of SCE after incubation in the presence of CY for

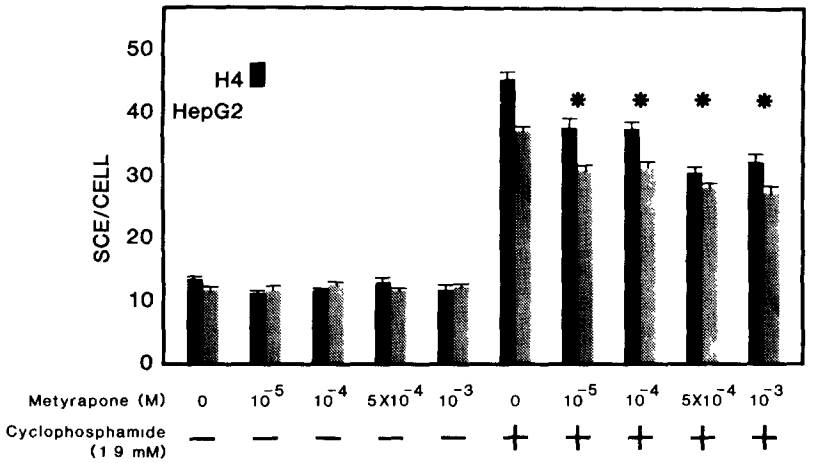

Fig 3 Effect of metyrapone on CY-induced SCE in HepG2 and H4 hepatoma cells. Cells were pretreated with indicated concentrations of metyrapone for 15 min Cultures were then exposed to metyrapone with or without cyclophosphamide (500 μg/ml, 1 9 mM) for their entire culture period (two cell cycles). Error bars indicate the standard error of the mean No significant differences were found between any of the SCE/cell frequencies for cultures incubated without cyclophosphamide There was a highly significant difference between hepatoma cultures treated with cyclophosphamide alone and all cultures treated with cyclophosphamide and metyrapone (* $P \leq 0\ 001$)

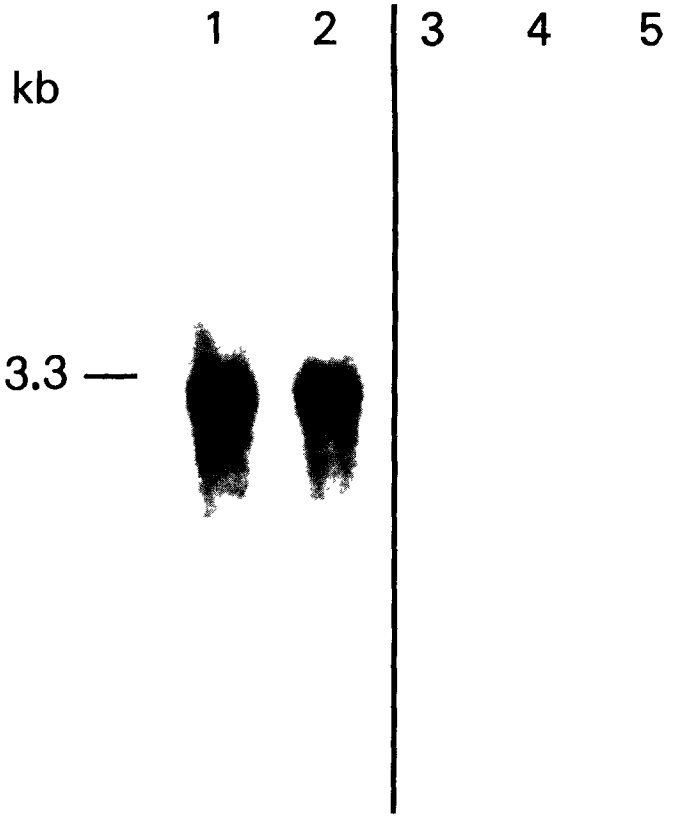

Fig 4 Relative levels of cytochrome P-450 transcripts Poly(A)RNA (5 μg) from a human liver sample (lane 1), HepG2 cells (lane 2), HL60 cells (lanes 3 and 4) and NIH 3T3 cells (lane 5) were fractionated on a denaturing formaldehyde gel, transferred to nitrocellulose and hybridized to a ^{32}P-labelled cDNA P-450 probe as described in Materials and Methods Ribosomal RNAs of 28s, 18s, 23s and 16s were used as standards to establish base size

two cell cycles All four cell lines showed similar concentration–response patterns after exposure to PM for two cell cycles These observations are of interest in light of the fact that cell cycle times differ dramatically for these cell lines [3], and that mutagen exposure times would therefore differ if the mutagenic agent were chemically stable in culture medium This is most likely not the case for PM, which is highly reactive. Therefore, it is likely that PM-induced DNA damage occurs shortly after the onset of incubation and the four cell lines sustain similar levels of damage This damage results in similar levels of SCE

When HepG2 and H4 cells were treated with CY for 60 min, similar numbers of SCE were again induced in both cell types This observation suggests that the two cell types have similar capacities for activating CY to DNA damaging forms since the studies with PM indicated that both cell types respond to equivalent levels of DNA damage with similar numbers of SCE However, since CY is stable for extended periods of time in the cell culture medium, one might expect higher SCE levels in HepG2 cells that were cultured in the presence of CY for 72 hr (two cell cycles) as compared to H4 cells which were cultured for 36 hr The observation that induced levels of SCE were similar in both cell types despite different CY exposure times may be the result of diminishing levels of activating enzymes It has been noted that acrolein, a product of CY metabolism, is capable of inactivating cytochrome P-450 enzymes [24, 25] Since both PM and acrolein are generated stoichiometrically [26], proportional levels of enzyme should be inactivated for each unit of CY that is activated If this were indeed the circumstance, our results suggest that HepG2 and H4 have similar levels of activating capacity

Studies with the cytochrome P-450 inhibitors SKF-525A and metyrapone provide indirect evidence that CY is indeed activated by this pathway in both hepatoma lines SKF-525A was able to reduce the level of CY-induced SCE when incubated for short time periods (30–60 min) When cells were incubated in the presence of both CY and SKF-525A for the entire culture period, no effect on SCE frequency was seen. Although the reason for the lack of effect is not known, this observation is consistent with the hypothesis that the cytochrome P-450 enzymes are inactivated in the process of converting CY to DNA damaging species [24, 25] Thus, SKF-525A might slow the rate of CY activation due to competitive inhibition [23], but the final amount of CY which could be converted would be limited by the presence of active P-450 enzymes. Lack of long-term inhibition is consistent with *in vivo* work where SKF-525A did not reduce the therapeutic efficacy of CY against experimental tumors [11, 27] SKF-525A also did not affect the level of CY-induced SCE in mouse bone marrow over the period of two cell division cycles [28]

Metyrapone provided a concentration-dependent decrease in the number of SCE induced by CY Since metyrapone apparently binds to or near the heme iron of cytochrome P-450 [29], this presents further evidence that these cell lines possess the major metabolic pathway for CY metabolism. As metyrapone did not compete for the CY activation site, this inhibitory effect was evident after incubation for the entire culture period, in contrast to the effect seen with SKF-525A after long-term exposure Since CY metabolism to genotoxic and cytotoxic forms was inhibited, the level of induced SCE was reduced and the possible inactivation of cytochrome P-450 enzymes by acrolein was not evident

Preliminary studies with indomethacin, an inhibitor of the cyclooxygenase activity of prostaglandin endoperoxidase, showed no alteration of CY-induced SCE in the hepatoma cell lines (data not shown) Indomethacin has been shown to reduce the levels of SCE induced by diethylstilbestrol [4] and benzidine*, chemicals known to be metabolized by prostaglandin endoperoxidase activity [30] in both HepG2 and H4 cells This suggests the utility of using inhibitors in the hepatoma cell lines to discern possible metabolic pathways Furthermore, this implicates the specificity of the cytochrome P-450 metabolism of CY in these hepatoma cells

Direct evidence for the existence of cytochrome P-450-associated gene activity was provided by Northern blot analysis. Poly(A)RNA isolated from HepG2 cells was hybridized to a rat cDNA P-450 clone, R17 As R17 is derived from an mRNA coding for a phenobarbital-induced form of P-450 [21] and CY is known to be activated by phenobarbital-inducible P-450 [22], the existence of this activation pathway is firmly established in HepG2

It is also interesting to note the unusually high molecular weight of the transcript coding for the human liver cytochrome P-450 detected in these experiments This large transcript suggests the presence of a long untranslated region within the mRNA which could function in a regulatory capacity This large size of the P-450 specific transcript has also been documented for certain mouse [31] and rat [32] cytochrome P-450s.

It is clear that short-term test systems are growing in usage and importance A test system composed of multiple cell lines as described here allows for the detection of genotoxicity by a chemical and its requirement for activation. Through the use of selective inhibitors and/or enhancers of a particular metabolic activity, the mechanism of activation may also be discerned. The cell lines examined in this report demonstrated the necessary requirement for cytochrome P-450-associated activation of CY to exert its genotoxicity, presumably via the demonstrated genotoxicity of PM. Finally, use of human-derived material, such as the HepG2 cell line, allows for assessment of action on the human genome By comparing the responses of cells from other species to the human response (e g. H4 versus HepG2), an evaluation of the appropriateness of selected species to represent human response to chemicals can be made

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