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Heterocyclic Amine Mutagenicity/Carcinogenicity: Influence of Repair, Metabolism, and Structure.

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Abstract: Cooking, heat processing, and pyrolysis of protein-rich foods induce the formation of structurally related heterocyclic aromatic amines that have been found to be mutagenic in bacteria, mammalian cells in culture and mice. All these compounds are potent mutagens and most are active below 1 ng/plate, in Ames/*Salmonella* tester strain TA1538 in the presence of S9 liver microsomal preparations from rat, mouse, or hamster. They are also potent in strains TA98, TA97, moderately active in TA1537, weakly active in TA100, and virtually inactive in TA1535 and TA102. Thus, they show powerful frameshift activity in reverting specific GC-rich sequences, but do not cause base substitution mutations or revert an AT-rich sequence. They are 100-fold less active in the uvrB+, repair-proficient strain TA1978, and in the case of IQ, cause insertions and large deletions not seen in TA1538.

Chinese hamster cells (CHO) were exposed to the mutagens in the presence of liver S9 from PCB-induced hamsters. Trp-P-2 and PhIP gave cytotoxic, mutagenic (hprt and aprt loci), and clastogenic (SCE and aberration) dose responses at 1 μg/ml. An excision repair-deficient cell line (UV-5) was more than twice as sensitive as the parental line (AA8)

for both mutagens. IQ was cytotoxic above 10 mg/ml; but there was no difference between the strains of differing repair capacity. Dose responses for mutagenesis and SCE occurred in UV-5 at 30 μg/ml, but chromosomal aberrations were not significantly increased. Genes for the expression of cytochrome P-450IA1 and IA2 were cloned into these CHO cells and cytotoxicity and mutagenicity were assessed. PhIP was a potent inducer of mutation at the APRT locus in the repair deficient cells expressing IA2. Repair competence reduced the response by approximately 5 fold. In contrast, Trp-P-2 was potent in the IA1 expressing cells that showed no activity for PhIP or IQ. The *in vivo* response of PhIP in the Mutamouse, a transgenic mouse having easily recoverable *lacz* genes for mutation analysis, was weak but positive in the pancreas.

Comparison of mutagenicity in *Salmonella* and carcinogenesis in rodents for both aromatic amines and the smaller subset of heterocyclic amines from heated foods, suggests a good correlation (R=0.66). This work suggests individual differences in repair and metabolism may have a big effect on both the type and amount of the mutagenic damage from these heterocyclic amines and that this mutagenicity may also correlate with the general carcinogenicity of the compound.

Concern about the role of diet in human cancer has prompted the search for compounds in common foods that may act as tumor initiators by producing somatic cell mutations. Analyses of pyrolyzed amino acids and proteins, and of cooked high-protein foods, led to the discovery of several classes of highly mutagenic heterocyclic amine compounds (1). Our

laboratory has been concerned with mechanisms of formation, the chemical identity, and the spectrum of genotoxicity of these mutagenic agents for more than 10 years (see (2) for review) and this report will highlight some of our recent findings on the mutagenicity, effects of DNA repair and relationship of mutagenicity to carcinogenicity for these compounds.

Bacterial Genetic Toxicology

1. *Microbiol Mutagenesis*

A number of food mutagens and analogs have been evaluated for microbial genotoxicity (3). All, with the exception of the PhIP isomers and AαC, are extremely potent mutagens, active below 1 ng/plate, in the Ames/*Salmonella* tester strain TA1538 in the presence of Aroclor induced liver S9 or induced microsomal preparations from rat, mouse, or hamster. They are also potent in strains TA98, TA97, moderately active in TA1537, weakly active in TA100, and virtually inactive in TA1535 and TA102. This is illustrated for 8-MeIQx in Fig. 1. PhIP and its 3-methyl isomer are 100 to 10,000 times less potent than the quinolines and quinoxalines in the responsive strains (4). But, in comparison to the carcinogen B[a]P, PhIP is still 10 times more potent. All of the heterocyclic amines tested show a preference for inducing frameshift mutation, but do cause some base substitution mutations at 20-100 fold higher doses. In addition, they are 100-fold less active in the uvrB+, repair-proficient strain TA1978 (5). The order of potency of the mutagens is 4-MeIQx > 4,8-DiMeIQx > IQ > MeIQx >PhIP > 3-MePhIP (4). MeIQ is among the most potent known *Salmonella* mutagens, in the same range of activity as the dinitropyrenes. IQ is also a potent mutagen in

two *Salmonella* forward-mutation assays: arabinose-resistance and 8-azaguanine resistance (6).

2. *DNA Sequence Analysis*

Analysis of the sequence changes in *Salmonella* after exposure to IQ, MeIQ, and PhIP show that all induced mutations in TA1538 and TA98 are caused by a single CG deletion in a run of CGs (7). Levine et al. (8) suggest that a C8dG adduct can cause a 2-base slippage in the positive strand in the 8 base run of alternating CGs in the *hisD* gene. When revertants induced by IQ are examined in the repair competent TA1978 strain, 48% of the sequence changes are large deletions and insertions compared to 0% in the *uvrB*$^-$ strain (see Table 1). Presumably the *uvrB* repair present in this strain can remove the lesions that normally lead to the -CG- deletion. PAHs like B[a]P behave much differently from the heterocyclic amines in this system, they give many more large deletions even when the uvrB repair system is not functional (7).

3. *Metabolism*

Recently, studies with S9 from CHO cells expressing only P450 1A2 derived from mice (P3 form) have shown quite remarkable mutagenicity for IQ and PhIP in TA1538 (9). In addition, *Salmonella* strains deficient for N-acetylation have shown loss of mutagenicity for IQ and MeIQx, but not for PhIP, suggesting differences in activation for PhIP compared to other heterocyclic amines (10, 11). Presumably PhIP can be activated by other phase II pathways such as by the sulfotransferase pathway (12).

Mammalian Toxicology

1. *In vitro mutagenicity and cytotoxicity*

Chinese hamster ovary cells (CHO) were exposed to various heterocyclic amine food mutagens in the presence of liver S9 from PCB-induced hamsters. Trp-P-2 and PhIP showed cytotoxic and mutagenic effects (hprt and aprt loci), and chromosome damage (SCEs and aberrations) at doses as low as 1 μg/ml (13). An excision-repair-deficient-cell line (UV-5) was more than twice as sensitive as the parental line (AA8) for both mutagens. IQ was cytotoxic above 10 mg/ml; but there was no difference between the strains having differing repair capacity. Dose responses for IQ mutagenesis and SCE formation occurred in UV5 at 30 μg/ml, but chromosomal aberrations were not significantly increased. No such responses occurred in AA8 up to 300 μg/ml. Data for MeIQ indicate that cytotoxicity occurs at approximately 150 μg/ml and above 300 μg/ml for MeIQx. Thus, the cytotoxicity and genotoxicity of these representative thermic mutagens in CHO cells are inversely proportional to their responses in *Salmonella* (13).

Fig 2 shows the mutagenic response at different doses of PhIP in the CHO cell strain UV5-P3-3. This strain expresses cytochrome P-450 1A2 (9). The average spontaneous frequencies in the aprt locus for the duplicate control cultures were 7.5×10^{-5}. The average survival at the highest dose was 2 percent. Cells were exposed for 48 h and showed a dose dependent increase in mutation frequency. IQ also showed a dose dependent increase in mutation frequency in these cells (9), but much lower mutation frequencies were achieved. UV5-P3-3 cells were reverted to repair competent by exposure to EMS. These cells still express cytochrome P-4501A2 but needed 10-20 fold higher dose to achieve significant mutational response. Clearly, this DNA

excision repair (analogous to human ERCC2) can lower the DNA damage and the resulting mutations, but it can be overcome at higher doses. It also must be remembered that the same compounds are carcinogenic, clastogenic and mutagenic (see below) *in vivo* in animals having normal DNA repair.

2. *DNA Binding*

In order to evaluate the response differences, *Salmonella* and CHO cells of each type were treated with radiolabeled Trp-P-2 and IQ. DNA was purified and counted for DNA adducts. Approximately 2 adducts per million bases were introduced with 5 mM Trp-P-2 in both *Salmonella* and CHO cells; the induced mutation frequencies were about equal at 25-30 per million surviving cells. This calculates to 2 X 10^8 adducts per mutational event. IQ induced the same adduct level in *Salmonella* at a concentration of 5 mM; induced mutations were about 5 per million cells or one-fifth of the level induced by Trp-P-2. However, in CHO cells, the IQ concentration required to induce two adducts per million bases was 132 mM; under these conditions the induced mutation frequency was 15 per million surviving cells, three-fold above that found with *Salmonella*. At the concentrations reported to give similar numbers of DNA adducts, the two agents have similar mutagenic efficiencies (mutations per adduct) in both assay systems (14).

3. *Metabolism*

The experiments described above do not explain the unexpectedly low mammalian genetic toxicity of IQ, but suggest either some difficulty in access of the active metabolite species to DNA in the mammalian system or metabolism is specifically different enough in CHO cells to effect the biological response compared to *Salmonella*. Recent studies suggest CHO

cells may lack the metabolic pathway [illegible] cetyltransferase (King, personal communication) that functions in *S. [illegible]lla.* strains TA98 and TA1538. In fact, the requirement for acetylation may explain the low biological activity of IQ and MeIQx in these cells.

It now seems clear from recent *in vitro* studies that acetylation is required for the formation of active electrophiles of IQ and MeIQx, but not necessarily for PhIP. DNA binding of N-hydroxy-PhIP depends primarily on bacterial sulfotransferase activity and not acetylation (12). *Salmonella* strains that have a deficiency in acetyltransferase activity have significantly lower mutation frequencies with IQ and MeIQx, but not with PhIP (15, 16). In collaboration with Dr. Josephy (Guelph Univ., Canada) we also showed that strains overexpressing acetyltransferase were more responsive to IQ, but not PhIP. Using the azido form of PhIP, Dr. Wild and his collaborators also showed this difference (10). It appears that N-OH intermediates of these amines have different requirements for conjugation to proximal electrophiles and these differences may explain variable responses in CHO cells and tissue specific carcinogenicity differences. These differences can be exploited in the "Mutamouse" for a better understanding of the mechanisms of metabolism and their effect on biological endpoints (see below).

4. *Mammalian In Vivo Toxicology*

In Aroclor-induced juvenile mice, the LD_{50} values for Trp-P-2 and IQ were 15 mg/kg and 150 mg/kg, respectively. Thus, Trp-P-2 was considerably more toxic than IQ *in vivo* which was similar to that observed *in vitro* with CHO cells. Dose responses were obtained in mouse bone marrow for SCEs: Trp-P-2 at 1.2-20 mg/kg, and IQ at 20-[illegible] mg/kg (17). In both cases there was a plateau response at high doses. Chromosomal aberrations (chromosome and

chromatid exchanges and deletions) in mouse bone marrow were dose-dependent with Trp-P-2 from 4-20 mg/kg; the response with IQ was negligible. Both PhIP and MeIQx induced SCEs *in vivo*. Aberrations were significantly increased with PhIP in peripheral blood lymphocytes. Thus, the reactive electrophiles are present extrahepatically and are able to react with DNA in peripheral tissues. This is supported by studies in mice and rats after longterm-feeding bioassays with PhIP (18, 19), as more nonhepatic tumors are seen with this heterocyclic amine- lymphocytic tissue, colon, and mammary glands are major sites. These conclusions are also supported by ^{32}P-postlabeling experiments from several laboratories (20, 21, 22) including our own (12). DNA binding is pronounced in the liver following exposure to IQ, MeIQx, and DiMeIQx, but PhIP shows higher binding in nonhepatic tissues.

5. Mammalian In Vivo Mutagenesis

The "Mutamouse" (Hazelton Laboratories) was used to examine tissue-specific mutagenesis following IP injection of PhIP. In this assay the mice were derived from a transgenic mouse having 40 copies of the lacZ gene in each cell (23). After waiting 7-14 days for the mutation to express the tissue of interest is removed and the DNA is extracted. The lacZ gene is then packaged in Lambda phage (packaging is quite easy because of the Cos sites constructed on both sides of the lacZ gene) and E. Coli C cultures are subsequently infected with the phage. The incubations are 18 h with X-GAL and IPTG for blue color formation of the functional lacZ gene plaques. Mutations knockout lacZ associated activity and give white plaques. The number of white plaques divided by the blue plaques is mutation frequency. We have singled out the pancreas and the liver to compare mutation frequencies with DNA binding.

Pancreas has shown exceptionally [illegible]duct formation with PhIP while liver is relatively lower. Table 2 sho[illegible]ne results for liver and pancreas. All the treatment doses showed a significantly higher than control mutation frequency (but no dose-response) in the pancreas, but no significant increase in the liver.

Mutagenicity verses Carcinogenicity

Hatch et al. (24) have examined the mutagenic and carcinogenic potency of 10 heterocyclic amines (thermic derived) and 24 aromatic amines for which both Ames/*Salmonella* and rodent carcinogenicity were available (Fig. 3). Potencies on mutagenic and carcinogenic scales were significantly correlated (R=0.66). Two structural features were found to modulate the two distinct biological responses. These features were the number of rings and the degree and placement of the methyl substitution at the carbon atoms. This data supports the use of short-term bacterial mutagenesis assays for evaluating health hazards for at least this class of chemical.

DISCUSSION

The heterocyclic amines clearly have an affinity for the C-8 of guanine and when tested in *Salmonella* strains TA98 and TA1538 cause a 2 base deletion in a run of C-Gs by a slippage mechanism. Clearly though, these compounds are genotoxins in many other systems such as 2 other bacterial test systems having different gene targets and base sequences, CHO cells with mutations, cell killing , and cytogenetic effects all being induced. In vivo responses are also strong for PhIP such as nonhepatic DNA binding, lacZ mutations in the pancreas of the "mutamouse", SCEs in the bone marrow.

The carcinogenicity data in both the rat, mouse, and monkey (25) suggest heterocyclic amines are strong carcinogens and among the most potent carcinogens in the aromatic amine class (Fig. 3).

The role of DNA repair is clearer from both the Salmonella and CHO cell data. Excision repair both in the Salmonella and CHO cells presumably removes adducts (most likely C-8 bulky adducts) resulting in 10-100 fold decrease in mutation frequency. It must be remembered that the *in vivo* mutation, cytogenetics and carcinogenesis responses are in animals that are normally not induced with cytochrome P-450 inducers or have repair deficiencies.

The types of base changes induced are dependent on DNA repair. When the *Salmonella* are uvrB$^-$ they accumulate adducts that lead to 2 base slippage. When the uvrB gene is functional, then the types of adducts are presumably different or at least the resulting mutation is more likely to be a large deletion or an insertion. It is possible these other types of lesions are formed at a low frequency, but are over shadowed by the C-G deletions in the repair deficient cells, but when the damage causing the C-G deletion is readily removed by the uvrB repair the smaller number of the other types of damage are apparent; as the mutation frequency also drops by 100 fold when uvrB is functional. In addition, the role of error-prone repair is also noteworthy. The heterocyclic amines seem to not be affected by this type of repair, but compounds like 4-aminobiphenyl and benzo[a]pyrene show 2 and 4 fold increases in their mutation frequency in TA98 (pKM101) repair plasmid) and changes in their mutation spectrum (8).

Finally, the comparison of carcinogenicity and mutagenicity is quantitatively significant. Heterocyclic amines are definitely more mutagenic

on a relative scale than they are ca... ...nic, but within a defined class of compounds (aromatic amines) they a... ...trong carcinogens as well.

TABLE 1

ROLE OF DNA REPAIR ON THE MUTATION SPECTRA IN THE AMES/SALMONELLA ASSAY

	TA1538 *uvrB-*		TA1978* *uvrB+*	
	Percent			
	-CG- deletions	Large Deletions and Insertions	-CG- deletions	Large Deletions and Insertions
IQ	100	0	58	42
B[a]P	63	37	0	100

*Mutation frequency is approximately 100-fold lower than TA1538. Methods used are described in Fuscoe et al (7).

TABLE 2

HETEROCYCLIC AMINE (PhIP) RESPONSE IN THE MUTAMOUSE

Dose	Plaques Rescued	Mutants	M.F. per 100,00 pfu
Pancreas			
0 mg/kg	727,361	6	0.82
1	205,445	16	7.78*
10	179,555	8	4.45*
25	203,950	14	6.86*
Liver			
0 mg/kg	160,312	5	3.11
1	130,647	3	2.29
10	244,930	5	2.04
25	283,990	14	4.92

*Statistically different from control (P<.05). Each dose point is the average of 3 mice injected IP with a single acute dose 9 days before sacrifice.

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KEY WORDS

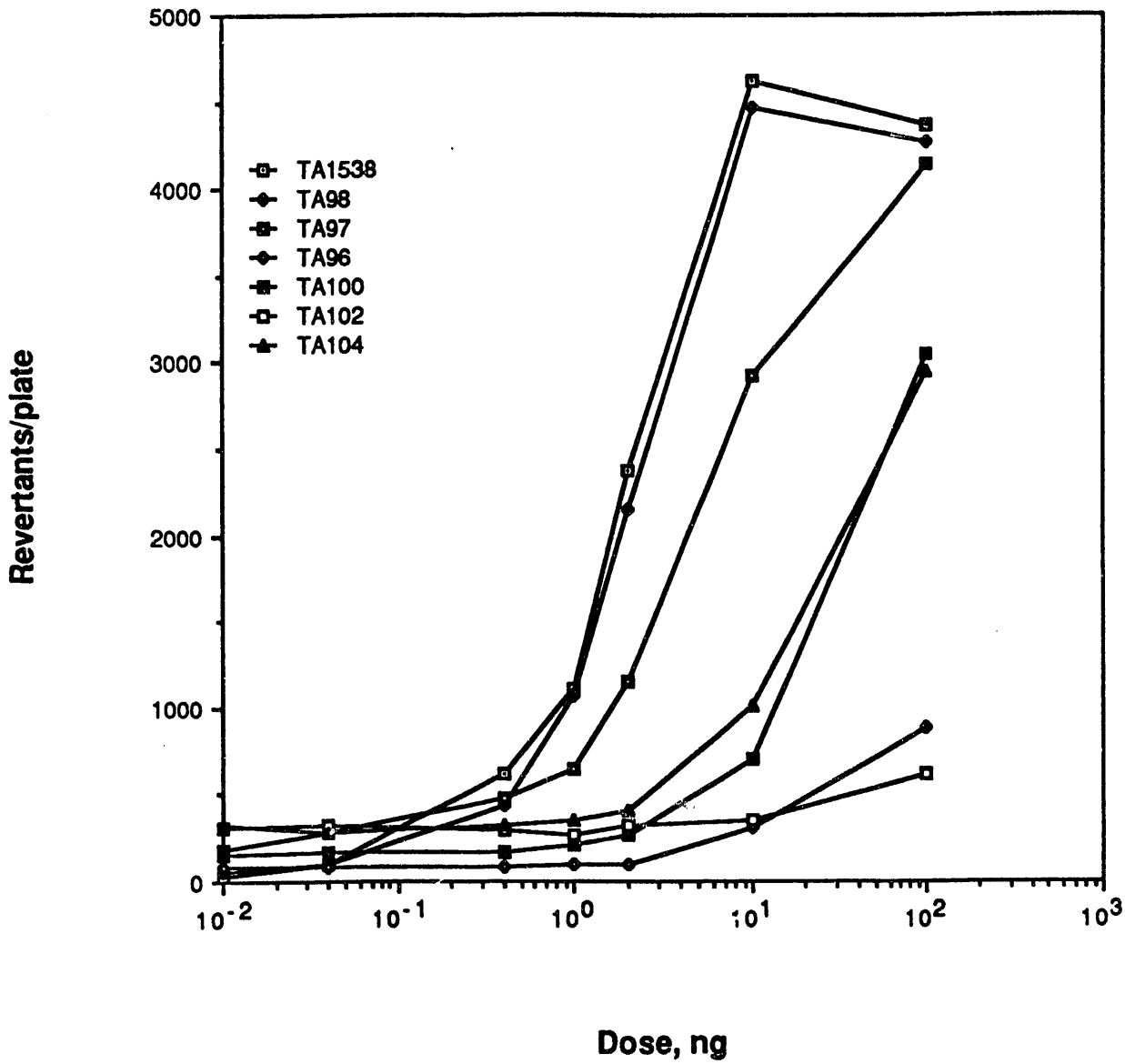
Heterocyclic amines, mutagenicity, Ames/Salmonella assay, Mutamouse, CHO cells, Carcinogenicity

FIGURE LEGENDS

Figure 1: Dose-response curves for 8-MeIQx in 7 Ames/Salmonella strains. The plate incorporation assay was used with Aroclor 1254-induced rat liver S9 at a concentration of 2 mg/plate. All points are the mean of duplicate platings.

Figure 2: Mutation frequency at the APRT locus induced by PhIP in CHO cell strain UV5-P3-3. The average spontaneous frequencies for the duplicate control cultures were 7.5 x 10^{-5}. The average survival at the highest dose was 2 percent. Cells were exposed for 48 h.

Figure 3: Plot of the mutagenicity and carcinogenicity of10 thermally produced heterocyclic amines and 23 aromatic amines. The X-axis is the log of the mutagenic potency in the Ames/Salmonella assay for either TA98 or TA1538. The Y-axis is the log of the carcinogenic potency in mice. Liver data is used in all cases except PhIP where lymphoma data is substituted. Correlation coefficient for this data is R=0.66.


5000
4000
3000
2000
1000
0
TA1538
TA98
TA97
TA96
TA100
TA102
TA104
Revertants/plate
10^{-2}
10^{-1}
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10^{2}
10^{3}
Dose, ng

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FIG 1

APRT mutation induced by PHIP in UV5-P3-3

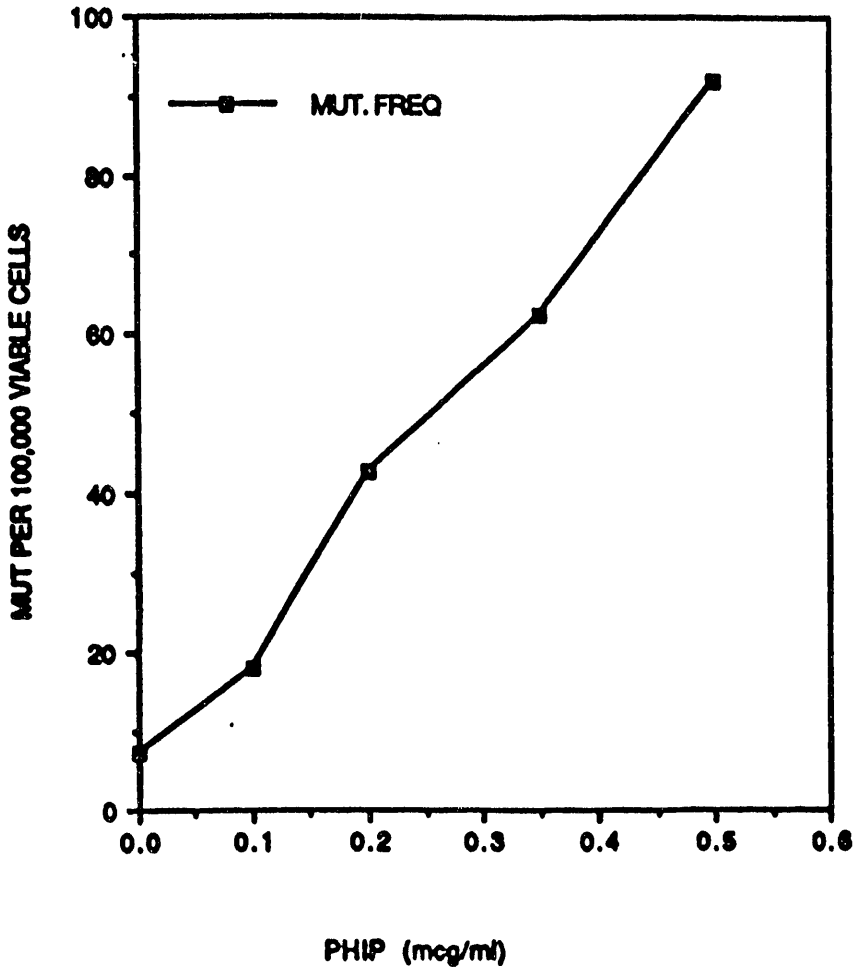

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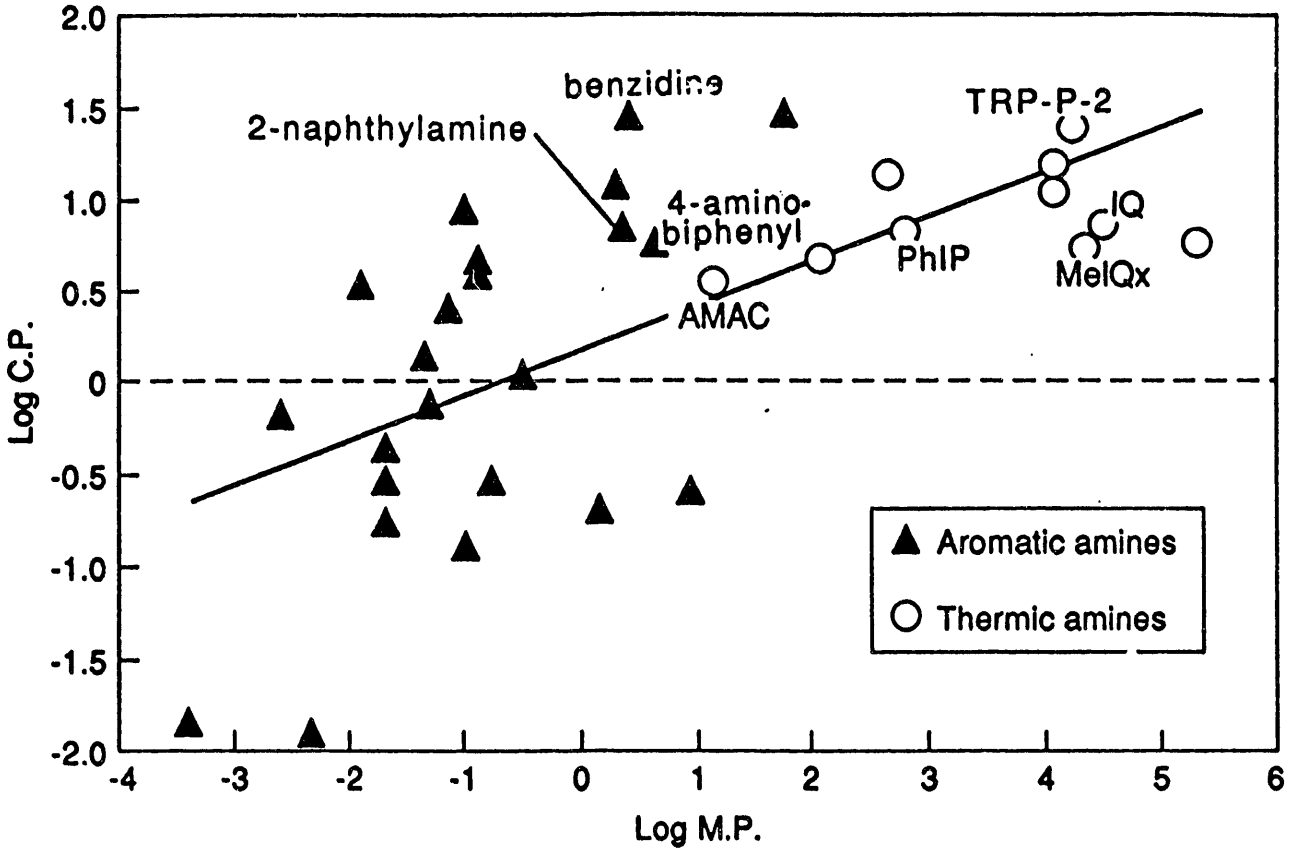
FIG 2

Carcinogenic vs Mutagenic Potency

Aromatic and heterocyclic amines



B-MK-1 (1-10-91) IL

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