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Short communication

Mutagenic Activity of a Mixture of Heterocyclic Amines at Doses below the Biological Threshold Level of Each

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The combined mutagenic effects of six heterocyclic amines (Trp-P-1, Trp-P-2, Glu-P-1, Glu-P-2, MeIQ, and IQ) at doses below the biological threshold level of each were investigated in the reverse mutation assay with *Salmonella typhimurium*. The lowest mutagenic doses of heterocyclic amines in TA1978P (*hisD3052*, *rfa*/pKM101) were 40 to 200 times the lowest mutagenic doses in TA98 (*hisD3052*, *rfa*, *uvrB*/pKM101), a strain deficient in nucleotide excision repair. The six heterocyclic amines were mixed at doses below the biological threshold that were mutagenic to TA98 but not to TA1978P. A significant increase in the number of revertants in TA1978P was observed with combined heterocyclic amines. The results suggest that DNA adduct formation at doses below the biological threshold level was additive, with the total amount reflecting the mutagenicity. We should consider the potential for additive effects of mutagens when we consider biological thresholds, because most of environmental mutagens exist in complex mixtures of chemicals.

Key words: biological threshold, heterocyclic amines, combined mutagenicity, *Salmonella typhimurium*

Introduction

While thresholds are reported for non-DNA targeting mutagens such as topoisomerase II inhibitors and spindle poisons, the absence of a threshold is assumed for DNA-targeting mutagens. The non-threshold model was first proposed for radiation, on the basis of the one-hit model of action for ionizing radiation, and then extended to chemical mutagens. It is based on the idea that the interaction of even a single molecule of mutagen with cellular DNA, at the right place and time, could lead to a genetic alteration (1). However, recent studies argue for the existence of a biologically meaningful threshold dose below which all induced DNA damage is repaired and consequently no induced mutations are detected. The contribution of DNA repair in the threshold dose responses is suggested by studies on alkylating agents (2,3) and other mutagens, such as 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone (MX), 4-nitroquinoline *N*-oxide, and furylfuramide (4), which showed that gene mutations are not induced in wild-type strains at concentrations that induce them in repair-deficient strains.

The concept of ''biological threshold'', originally defined by Seiler as ''Real (or biological) threshold'' (5), is ''the chemical is present, and can interact with the target, but no adverse consequence is induced'' (6). I used the term of ''biological threshold'' in this paper to explain the dose below which all induced DNA damage was repaired and consequently no reverse mutations were detected in the DNA repair-proficient strain (4). Induction of DNA damage could be proved by evidence that mutagenicity at the dose was detected in the corresponding DNA repair-deficient strain (4). Assuming that a biological threshold indeed exists for DNA-targeting mutagens, the question arises as to whether multiple mutagens at individual threshold doses would exhibit additive mutagenicity. This is an important question, because most environmental exposures involve complex mixtures of chemicals. Combined effects were reported for five or ten heterocyclic amines administered together at low doses to F344 rats in the quantitative analysis of glutathione S-transferase placental form (GST-P) positive hepatic foci development in a medium-term bioassay system for carcinogenesis (7,8), and DNA adduct formation in the same system measured by the ^{32}P-postlabeling method was demonstrated to be additive (9). We therefore may need to take into account the combined effect of mutagens at individual thresholds, when we consider biological thresholds. To address this issue, I compared the mutagenic effects of 6 individual heterocyclic amines in a nucleotide excision repair-deficient strain TA98 of *Salmonella typhimurium* and the wild-type strain TA1978P. Then I tested the 6 mutagens mixed together at doses below the mutagenic threshold of each for TA1978P.

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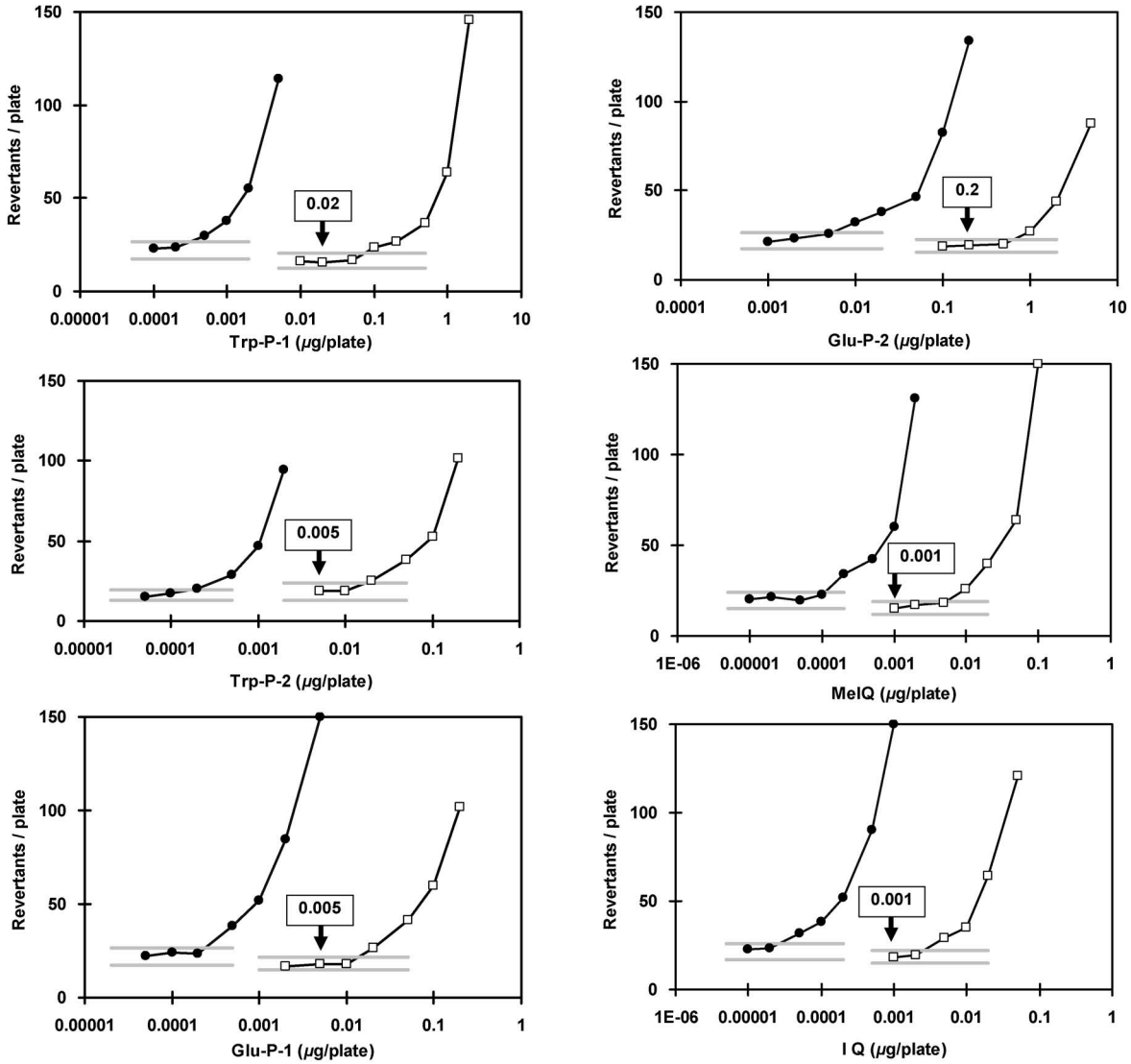

Fig. 1. Comparison of mutagenic response to heterocyclic amines (Trp-P-1, Trp-P-2, Glu-P-1, Glu-P-2, MeIQ, and IQ) in strains TA98 (*uvrB*) and TA1978P ($uvrB^+$) in the presence of S9mix. Data are the means of 4 plates for each dose of mutagen. Two gray horizontal lines indicate one SD above and below the mean of 8 concurrent control plates. Arrows indicate the dose selected for assaying combined mutagenicity. (closed circle), TA98; (open square), TA1978P.

Materials and Methods

Chemicals, bacterial strains and media: 3-Amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-1), 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-2), 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), and 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MelQ) were purchased from Wako Pure Chemical Industries Ltd., (Tokyo). 2-Amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazble (Glu-P-1) and 2-aminodipyrido[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-2) were a gift of Dr. Mie Akanuma (IET, Tokyo). Heterocyclic amines were dissolved in dimethylsulfoxide (DMSO). *S. typhimurium* strains TA1978P (*hisD3052*, *rfa*/pKM101) and TA98 (*hisD3052*, *uvrB*, *rfa*/pKM101) were used for the reverse mutation assays. TA1978P was constructed by conjugation between TA1978 (10) and *Escherichia coli* WP2*uvrA*/pKM101 (11,12). TA1978 and TA98 were obtained from Prof. Bruce N. Ames (CA, USA). Bacteria were cultured in nutrient medium (Oxoid, No.2) at 37°C with shaking. Minimal glucose agar plates consisted of Vogel-Bonner E medium supplemented with 2% glucose and 1.5% agar (10). Top agar (0.6% agar and 0.5% NaCl) contained 0.05 mM D-biotin and 0.05 mM L-histidine. S9 mix for metabolic activation consisted of 10% (v/v) S9 fraction, 8 mM $MgCl_2$, 33 mM KCl, 5 mM glucose-6-phosphate, 4 mM NADH, 4 mM NADPH, and 100 mM sodium phosphate buffer (pH7.4). The S9 fraction (Oriental Yeast Co., Tokyo) had been prepared from livers of male SD rats pretreated with phenobarbital and 5,6-benzoflavone

as cytochrome P-450 inducing agents. S9 mix was prepared just prior to experiments by combining S9 fraction and the cofactor solution (Cofactor A, Oriental Yeast Co.).

Reverse mutation assay: I conducted the His^+ reversion assay with *S. typhimurium* strains TA98 and TA1978P, because heterocyclic amines are known to induce predominantly -2 frameshift mutations rather than base-substitutional mutations (13–15). The bacteria were precultured overnight in nutrient broth. Aliquots of 0.1 mL of bacterial culture and 0.005–0.02 mL of a mutagen solution were added to 0.5 mL of S9mix. The total volume of DMSO in each test tube was adjusted to 0.1 mL. After preincubation at 37°C for 20 min, treated cells were poured onto MG agar plates with 2 mL molten top agar. His^+ revertants were counted following 48 h incubation at 37°C. Experiments investigating individual dose-response curves included 4 plates for each dose of mutagen and 8 plates for the solvent control. Experiments investigating combined effects were conducted with 5 plates (Exp. 1) or 7 plates (Exp. 2) for the mutagen-treated group and the solvent control. The Dunnett test was used for statistical analysis.

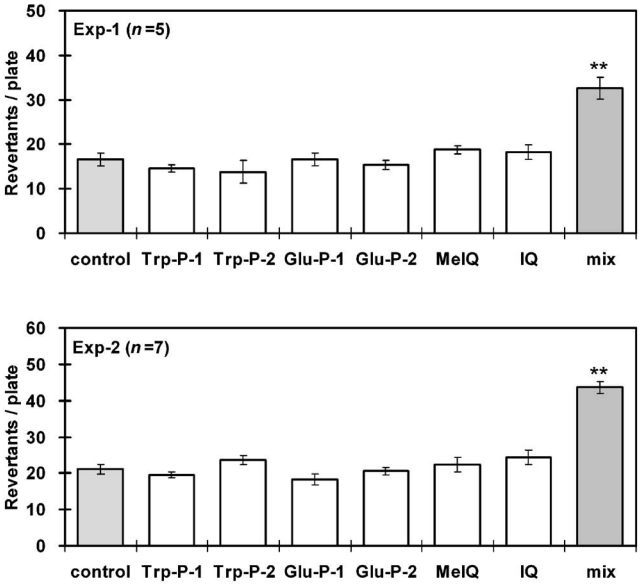

Fig. 2. Additive effect of heterocyclic amines at doses below the biological threshold in TA1978P. Dose of Trp-P-1, Trp-P-2, Glu-P-1, Glu-P-2, MeIQ, and IQ were 0.02, 0.005, 0.005, 0.2, 0.001, and 0.001 μg/plate, respectively. Data are the means of 5 plates in experiment 1 and 7 plates in experiment 2. Error bars mean standard error (SE). (darkgray bar), mixture of 6 heterocyclic amines; (gray bar), solvent control; **, $P<0.01$ (Dunnett test).

Results and Discussion

To investigate the contribution of DNA nucleotide excision repair capability on mutagenesis induced by heterocyclic amines, I tested the compounds in *S. typhimurium* strains TA98 (*uvrB*) and TA1978P, and compared the mutagenic response. Fig. 1 shows the dose-response curves of Trp-P-1, Trp-P-2, Glu-P-1, Glu-P-2, MeIQ, and IQ in TA98 and TA1978P. The response at low doses is hidden within the background variability. Trp-P-1 increased the number of His^+ revertants above the spontaneous level starting at 0.0005 μg/plate in TA98, and at 200 times that (0.1 μg/plate) in TA1978P (Fig.1). IQ induced His^+ revertants starting at 0.00005 μg/plate in TA98 and at 0.005 μg/plate in TA1978P. The lowest mutagenic doses of Trp-P-2 (0.02 μg/plate), Glu-P-1 (0.02 μg/plate), Glu-P-2 (1 μg/plate), and MeIQ (0.01 μg/plate) in TA1978P were 40 to 100 times the lowest mutagenic doses in TA98. These results indicate that the existence of a biological threshold below which mutagenicity is not evident in TA1978P, a strain proficient in a nucleotide excision repair function. I selected a dose below the biological threshold in TA1978P for each mutagen (0.02 μg/plate for Trp-P-1, 0.005 for Trp-P-2, 0.005 for Glu-P-1, 0.2 for Glu-P-2, 0.001 for MeIQ, and 0.001 for IQ). Since the compounds at those doses were highly mutagenic to TA98, they presumably provoked DNA damage in TA1978P that was repaired. When the 6 heterocyclic amines were combined at the above doses and assayed the mutagenicity with TA1978P, they induced a significant increase in the number of revertants with reproducibility (Fig. 2). These results suggest that the DNA damage (adduct formation) induced by subthreshold levels was additive and the total amount of DNA damage reflected collective mutagenicity. Therefore, the biological threshold concept (4–6) for mutagenic compounds may not be applicable if only individual thresholds are determined, and that is worth particular attention when evaluating the mutagenicity of our environmental mixtures. Shimoi *et al.* (16) reported the inhibitory effect of heterocyclic amines on the nucleotide excision repair activity in *E. coli* in the absence of S9mix. However, the doses of heterocyclic amines used in their experiments were in the order of 10–100 μg/plate. Since the present study was conducted at very low doses of 0.001–0.2 μg/plate in the presence of S9mix, the inhibitory effect would not affect on the mutagenicity. It is obvious that the contribution of inhibitors on DNA repair systems is another important factor when we consider biological threshold (17). Further investigations are necessary to determine whether additive effects at biological threshold level are the rule in combinations of other types of mutagens, such as direct-acting and structurally different mutagens.

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