**Preliminary Communication**

Tryptophan Pyrolysis Products, Trp-P-1 and Trp-P-2 Induce Apoptosis in Primary Cultured Rat Hepatocytes

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The cytotoxicity of heterocyclic amines, dietary carcinogens derived from cooked foods, to primary cultured rat hepatocytes was studied. A tryptophan pyrolysis product, 3-amino-1,4-dimethyl-5H-pyrido[4,3-b]indole (Trp-P-1) was the most cytotoxic of 11 compounds tested. Trp-P-1 was found to induce apoptosis as measured by morphological changes in nuclear chromatin and internucleosomal DNA fragmentation. 3-Amino-1-methyl-5H-pyrido[4,3-b]indole (Trp-P-2) showed a moderate apoptotic effect, and other compounds had a similar but weaker effect.

**Key words:** heterocyclic amine; tryptophan pyrolyzate, Trp-P-1; apoptosis; hepatocytes; rat

Heterocyclic amines have been isolated from the charred portions of cooked food and found to be potent bacterial mutagens. They were carcinogenic in animals when administered orally, and this carcinogenicity has been demonstrated most frequently in the liver (reviewed in references 3 and 4). It is well known that heterocyclic amines require microsomal activation to form DNA adducts and exert their genotoxicity. Harman and norharman, which are not mutagenic by themselves either with or without microsomal activation, have also been detected in tryptophan pyrolysates (reviewed in references 7 and 8). The genotoxic potential of heterocyclic amines has also been demonstrated using cultured mammalian cells. For example, not only heterocyclic amines but also harman and harmine enhanced UV-induced chromosome aberrations in CHO cells. In the same study, Trp-P-1 and Trp-P-2 did not require microsomal activation for the enhancement of chromosome aberrations, and this co-mutagenic effect was considered to arise from inhibition of DNA excision repair. These results indicate that both Trp-P-1 and Trp-P-2 were shown to act as direct genotoxic agents by themselves in CHO cells.

Previously, we showed that Trp-P-1 and Trp-P-2 were cytotoxic to primary cultured rat hepatocytes. 2-Acetylamino-1-fluorene, a chemical carcinogen having a Trp-P-1 or Trp-P-2 related chemical structure, also showed a moderate cytotoxic effect. A recent report demonstrated that 2-acetylaminofluorene induced a mitogenic response with an increase in the number of apoptotic cells in the portal areas of the liver when administered to Fisher 344 rats by gavage. Induction of apoptosis was also detected in parenchymal cells. We therefore hypothesized that heterocyclic amines and their related analogues induce apoptosis in mammalian cells after a genotoxic effect, such as aberration of chromosomes, which arise from exposure to these compounds. However, few studies have been published to find whether exposure to heterocyclic amines is sufficient to induce apoptosis in mammalian cells.

In this study, we have investigated the cytotoxicity of heterocyclic amines and their related compounds to primary cultured rat hepatocytes. Trp-P-1 was found to be the most toxic compound among seven heterocyclic amines and four analogue compounds. In addition, we observed bio-markers for apoptosis, including chromatin condensation and fragmentation, after treatment with Trp-P-1.

Parenchymal hepatocytes were isolated from male Wistar rats (body weight 200–250 g, Japan SLC Inc., Shizuoka, Japan) by in situ perfusion of liver with collagenase solution by the method of Tanaka et al. Isolated hepatocytes were suspended at a concentration of 5 x 10^6 cells/ml in William’s medium E with 100 mM insulin, 100 mM dexamethasone, kanamycin (100 mg/l), aprotinin (10 KIU/ml), and 5% fetal bovine serum. Cells were seeded on the plastic multiplates or dishes (Becton Dickinson Co., Ltd., Franklin lakes, NJ) pre-coated with collagen, then cultured in an atmosphere of 95% air-5% CO2 at 37°C for 2 h. Cell treatments were done in dishes by addition of stock solutions of heterocyclic amines in dimethyl sulfoxide (DMSO) directly to the culture medium. Parallel dishes received a vehicle alone to obtain control samples (concentration of DMSO was maximally 0.1% v/v). The following commercially available heterocyclic amines were used: Trp-P-1 (acetate form), Trp-P-2 (acetate form) and 1-methyl-9H-pyrido[3,4-b]indole (harmol) from Wako Pure Chemical Industries (Osaka, Japan); 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP); 2-amino-3-methylimidazo[4,5-f]quinoline (IQ), 2-amino-3,4-dimethylimidazo[4,5-f]quinoline (MeIQ), 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx), and 2-amino-9H-pyrido[2,3-b]indole (A[a]C) from Dr.桷ess Research Chemicals (Downsvlew, Ontario, Canada); 9H-pyrido[3,4-b]indole (norharmane) from the Sigma Chemical Company (St. Louis, MO); and 1-methyl-9H-pyrido[3,4-b]indole (harmane, hydrochloride form) and

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7-methoxy-1-methyl-9H-pyrido[3,4-b]indole (harine, hydrochloride form) from Nacalai Tesque (Kyoto, Japan).

Cytotoxicity of heterocyclic amines and their related compounds to primary cultured hepatocytes was estimated by measuring cell viability and by a lactate dehydrogenase (LDH) leakage test. After treatment with test compounds for the indicated times in each figure, cell viability was measured using an MTT test.\(^{10}\) For the LDH leakage test, culture medium was stored and cells were homogenized with 0.05 M phosphate buffer (pH 7.4) by ultrasonication. LDH activity was measured both in the culture medium and in cell homogenates.\(^{17}\) LDH leakage is expressed as the percentage of activity in medium against the sum of the medium and intracellular activities. As shown in Fig. 1, 60 μM of Trp-P-1 strongly suppressed cell viability to 7%, and Trp-P-2 also suppressed it to 50% 6 h after treatment. Other heterocyclic amines, PhIP, IQ, MelQ, MelQx, and AfαC did not show any cytotoxicity to hepatocytes. Harmine, harmarne, norharmane, and harmol slightly reduced cell viability, but not significantly. Similar results were observed at 12 and 24 h after treatment, except that harmol significantly decreased cell viability to 65% by 24 h (data not shown). These data indicate that only Trp-P-1 and Trp-P-2 have a potent cytotoxicity to cultured hepatocytes. Accordingly, the dose-response and time-course effects of Trp-P-1 and Trp-P-2 on cell viability and LDH leakage were further investigated. Trp-P-1 reduced cell viability and increased LDH leakage at 30 μM and more, while Trp-P-2 decreased cell viability at 60 μM and more and caused LDH leakage at 120 and 240 μM (Fig. 2A). At 60 μM, Trp-P-1 and Trp-P-2 decreased cell viability by 2 and 6 h, respectively, in a time-dependent manner (Fig. 2B). Trp-P-1 caused a significant increase in LDH leakage 4 h after treatment, but Trp-P-2 did not. Other chemicals did not cause any LDH leakage 24 h after treatment at 240 μM.

With regard to the cytotoxicity to hepatocytes after treatment with eleven compounds tested, the degree of toxic activity was as follows: Trp-P-1 > Trp-P-2 > harmol > others. At least an imidazoquinoline or imidazopyridine ring did not contribute to the toxicity, because IQ, MelQ, and PhIP were not toxic to hepatocytes. A key structural feature of heterocyclic amines required for the induction of cytotoxicity is a pyridoindole ring with an amino group. Among Trp-P-1, Trp-P-2, and AfαC, the toxicity was dependent on the number of methyl groups. Interestingly, the cytotoxic activity did not correlate with mutagenicity, based on the fact that the mutagenicity of Trp-P-1 was weaker than that of Trp-P-2,\(^{4}\) and on the result that harmane and norharmane are comutagens but not mutagens by themselves.\(^{7,8}\) Carcinogenicity of Trp-P-1 is stronger than that of Trp-P-2 in liver of Fisher 344 rats.\(^{10}\) It is, therefore, difficult to elucidate the relationship between the toxicity and chemical structures of heterocyclic amine type chemicals.

We next investigated the ability of heterocyclic amines and their related compounds to induce apoptosis. To assess oligonucleosomal laddering of cellular DNA, we prepared DNA from hepatocytes and analyzed it by a modification of the electrophoretic method described previously.\(^{19}\) Briefly, hepatocytes (2 × 10⁵ cells) were treated with the various concentrations of test compounds for 6 h, then lysed in a 0.2 ml TE buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA) containing 0.5% sodium dodecyl sulfate. The resulting lysate was incubated with 0.5 mg/ml RNase A at 50°C for 30 min and then with 0.5 mg/ml proteinase K at 50°C for 60 min. After the addition of 0.5 M NaCl and 1 mM EGTA at the final concentrations, DNA was precipitated with 50% isopropanol at −20°C overnight. DNA precipitate was obtained by centrifugation at 15,000 rpm for 20 min,
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Fig. 3. Effects of Heterocyclic Amines and Their Related Compounds on DNA Fragmentation in Hepatocytes.

Hepatocytes were treated with 60 μM of heterocyclic amines for 6 h. DNA fragmentation was analyzed by 2% agarose gel electrophoresis and showed a typical pattern.

washed with 70% ethanol, and resuspended in TE buffer. DNA was resolved by 2% agarose gel electrophoresis in a TBE buffer (89 mM Tris-HCl, 89 mM borate, and 2 mM EDTA), then stained with ethidium bromide and visualized by UV. Trp-P-1 caused the characteristic DNA laddering of apoptosis in a dose-dependent manner by DNA gel electrophoresis (Fig. 3, lanes 2–5). DNA fragmentation was observed time-dependently at 30 μM Trp-P-1 (data not shown). Trp-P-2 also showed DNA laddering at 60 μM (lane 6) but did not show laddering at lower concentrations (data not shown). The ratio of DNA fragmentation by Trp-P-1 and Trp-P-2 to that of control was similar to the DNA laddering pattern; In the case of by 60 μM Trp-P-1 and Trp-P-2, DNA fragmentation was increased to approximately 15- and 7-fold that of the control, respectively (data not shown). Other compounds caused weak DNA laddering (Fig. 3, lanes 7–15). Among the compounds tested, therefore, Trp-P-1 most strongly induced apoptosis in hepatocytes.

Further confirmation that Trp-P-1 induces apoptosis was given by the morphological appearance of hepatocytes stained with Hoechst 33258 as described previously with a slight modification. Briefly, hepatocytes were cultured on a plastic cover slip and treated with or without 30 μM Trp-P-1. Cells were fixed with 1% glutaraldehyde in PBS for 30 min, washed in PBS twice, and stained with 1.5 μg/ml Hoechst 33258 for 30 min. Thereafter, morphological changes in the nuclear chromatin of apoptotic cells were observed by fluorescence microscopy. The nuclei of control cells stained uniformly with this dye, indicating that the nuclei were intact and viable (Fig. 4A). Conversely, treatment of the cells with Trp-P-1 (30 μM for 6 h) caused nuclear fragmentation and condensation (Fig. 4C). In parallel, phase contrast microscopy showed that the cell structures of Trp-P-1-treated hepatocytes were disintegrated into large blebs with diameters of 5 to 10 μm (compare Fig. 4D with B).

These results taken together clearly showed apoptotic hallmarks such as DNA fragmentation and morphological changes in nucleus with drastic reduction of cell viability after treatment with tryptophan pyrolys products. This is the first report to demonstrate Trp-P-1 and Trp-P-2, well known dietary hepatocarcinogen, induce apoptosis in primary cultured rat hepatocytes.

Trp-P-1 and Trp-P-2 are known to be metabolized to their N-hydroxy forms by cytochrome P450s, and to subsequently form DNA adducts. It is important to know whether Trp-P-1 and Trp-P-2 require microsomal activation for the apoptotic process they induced. Under our experimental conditions, hepatocytes from untreated rats (i.e., rats did not receive any treatment with chemical) will maintain the slight amounts of cytochrome P450s, even if the amounts would be reduced during preparation and culture of the cells. We, then, tried to measure N-hydroxy-Trp-P-1 by the method reported previously, but could not detect the N-hydroxy form in 2-h-cultured hepatocytes from untreated rats (data not shown). It would appear, therefore, that Trp-P-1 might cause apoptosis without microsomal activation by cytochrome P450s, although it still remains possible that cytochrome P450s are involved in Trp-P-1-induced apoptosis. Recently, the cytotoxicity and DNA damage-repair of N-nitrosodialkylamines were investigated in cultured hepatocytes isolated from untreated, phenobarbital- and pyridine-treated rats. The cytotoxicity and DNA damage-repair were involved
in the content of cytochrome P450s in hepatocytes. On the other hand, Trp-P-1 enhanced the induction of mutations and chromosome aberrations by UV in CHO cells without microsomal activation. Under cell-free conditions, Trp-P-1 is known to change DNA conformation by intercalation, and to causes inhibition of T4 endonuclease V and topoisomerase I, suggesting that these effects would be partly related to the inhibition of DNA excision repair and would enhance UV-or chemically induced mutagenic activity. In addition, harman and norharman have shown weak effects but PHIP has not, indicating that these effects are specific to tryptophan pyrolys products. Thus, tryptophan pyrolys products, such as Trp-P-1 and Trp-P-2 are likely to inhibit DNA repair by changing DNA conformation even without microsomal activation.

Recently, camptothecin, which induces apoptosis in various cell types through the inhibition of topoisomerase I, has been shown to sequentially induce ceramide synthesis and the activation of the caspase cascade. Therefore, the inhibition of topoisomerase I by tryptophan pyrolys products will also act as a trigger for apoptosis and will cause ceramide synthesis and/or the activation of the caspase cascade in the same manner as camptothecin. Further studies will be needed to elucidate the exact mechanism for induction of apoptosis by tryptophan pyrolys products.

References


