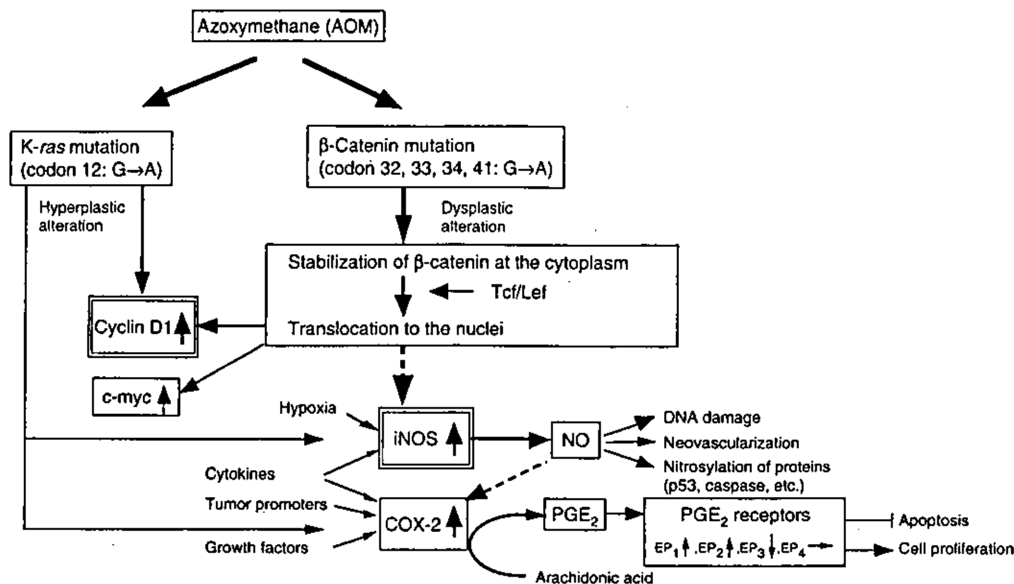

Fig. 3. Gene mutations and altered expression of proteins in rat colon carcinogenesis induced by AOM.

staining for iNOS was clearly observed in the carcinoma epithelial cells,[37] predominantly at the luminal surfaces of carcinoma cells forming glandular patterns, but not in moderately differentiated adenocarcinoma cells not forming clear glandular patterns, implying the involvement of factors other than alteration of β-catenin alone. Since some colon cancer cell lines are known to express iNOS on cytokine treatment,[38] cytokine receptors and/or subcellular components present in well-differentiated cells might be contributing to the iNOS expression. The results therefore suggest that increased expression of iNOS could be related to the altered localization of β-catenin in the presence of other factors.

Furthermore, we examined the relation between K-*ras* and increased expression of iNOS in a cell culture system (Fig. 2). When IEC-6 rat intestinal epithelial cells were transfected with K-*ras* mutant cDNA, iNOS expression mediated by interleukin-1β (IL-1β) or lipopolysaccharide (LPS) was markedly elevated, whereas transfection of the control vector or wild-type K-*ras* cDNA did not result in enhanced iNOS expression.[39] These results suggest that K-*ras* with an activating mutation can elevate iNOS expression in the presence of inflammatory stimuli, and thereby presumably promote tumorigenesis.

2.2 COX-2 and prostanoid receptors

As summarized in Table 2, COX-2 expression in stromal cells is a feature of normal colon mucosa and ACF, but is clearly increased in the stromal elements of adenomas and adenocarcinomas. On the other hand, COX-2 expression in epithelial cells is negative in normal mucosa and ACF, slight in adenomas, but frequent in adenocarcinomas, being located in the cytoplasm and nuclear membranes of carcinoma cells forming glandular patterns. COX-2 expression in both stromal and epithelial cells is particularly high in large carcinomas, suggesting that this protein enhances tumor growth.[29] COX-2 overexpression has been demonstrated to render tumor cells resistant to apoptosis and to stimulate neovascularization, thus conferring a survival advantage.[40-42]

Some evidence of an involvement of the Wnt/Apc/β-catenin/Tcf pathway in COX-2 expression has been presented.[43-45] However, our results indicate that β-catenin alteration is not in itself sufficient for induction of COX-2. It has also been reported that K-*ras* mutations and/or activation increased expression of COX-2, but β-catenin mutations did not, in AOM-induced rat colon tumors.[46] In our studies, a clear relationship between K-*ras* mutations and COX-2 expression was not evident, but both were frequent in relatively large adenocarcinomas. Like iNOS, preferential expression of COX-2 could be demonstrated in well-differentiated carcinoma cells forming glandular patterns,[29] and it has been reported that NO enhances activity and expression of COX-2 in several cell lines.[47-50] A causal relationship between the two is therefore conceivable.

In colon cancers, PGE_2 synthesis is generally elevated,[51,52] and it is very likely that this would enhance carcinogenesis. Indeed, administration of PGE_2 enhanced colon carcinogenesis in rats treated with AOM through induction of cell proliferation and reduction of apoptosis.[53] Prostanoids exert their biological actions through binding to their specific membrane receptors and there are four PGE_2 receptor subtypes, EP_{1-4}. Using prostaglandin E receptor subtype-knockout mice, the roles of these receptors in colon carcinogenesis have been investigated in our laboratory.[54-56] Deficiencies of PGE_2 receptors EP_1 and EP_4 caused a decrease in ACF formation in the colons of mice treated with AOM.[54,55] Deficiencies of EP_2 and EP_3 did not affect AOM-induced ACF formation,[55] but Sonoshita *et al.* reported that double knockout of *Apc* and *EP_2* genes decreased intestinal polyp development.[57] On the other hand, deficiency of EP_3 enhanced colon tumor formation induced by AOM.[56] These observations suggest that EP_1 and EP_4 are promotive in early steps of colon carcinogenesis, and EP_2 and EP_3 play promotive and suppressive roles, respectively, in later steps. RT-PCR analysis of mRNA expression of PGE_2 receptors demonstrated up-regulation of EP_1 and EP_2 and down-regulation of EP_3 in AOM-induced rat and mouse colon cancers. EP_4 mRNA was constantly expressed in normal mucosa and tumors.[56]

2.3 Others

Bissonnette *et al.* reported that mutations in either K-*ras* or β-catenin increase expression of cyclin D1 in AOM-induced rat colon tumors.[46] It is well known that *cyclin D1* is a target gene of β-catenin/Tcf signaling and its increased expression has been observed in human colon cancers.[58,59] Another target of β-catenin/Tcf signaling is c-myc mRNA expression, which has also been reported to be increased in the early stage of colon carcinogenesis in rats treated with AOM.[60,61]

Conclusion

From the above observations, we have constructed a tentative schema for AOM-induced colon carcinogenesis, as shown in Fig. 3. AOM induces G-to-A transitions in the K-*ras* and/or *β-catenin* gene. K-*ras* mutations at codon 12 may contribute to induce hyperplastic changes, while *β-catenin* mutations seem to be involved in generation of dysplastic lesions. Mutated K-ras activates the MAPK and PI3K pathways, and then up-regulates cyclin D1 and COX-2, also enhancing iNOS expression in the presence of inflammatory stimuli. *β-Catenin* mutations stabilize β-catenin protein in the cytoplasm and activate transcription of the targets of β-catenin/Tcf signaling, such as cyclin D1 and c-myc. β-Catenin alteration may also be involved in increased expression of iNOS. NO produced by iNOS causes DNA damage and neovascularization, while activating COX-2. Overexpression of COX-2 produces excess prostaglandins, and causes an increase in cell proliferation and decrease of apoptosis, to some extent mediated by PGE_2 receptor subtypes EP_{1-4}.

The data obtained in these studies point to particular mechanisms of colon carcinogenesis and indicate that further investigations of cross-talk between the Wnt/β-catenin/Tcf pathway and the K-ras/MAPK pathway in colon carcinogenesis are warranted.

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Identification of cytochrome P-450s involved in the formation of APNH from norharman with aniline

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Abstract

Mutagenic 9-(4′-aminophenyl)-9*H*-pyrido[3,4-*b*]indole (aminophenylnorharman, APNH), formed from norharman and aniline in the presence of S9 mix, is thought to be accountable for the co-mutagenic action of norharman. Our previous studies suggest that cytochrome P-450s (CYPs) are involved in the generation of APNH. In order to identify the responsible CYP species in the present study, norharman (8 mg) and aniline (4 mg) were incubated with individual recombinant human CYPs (2 nmol) at 37 °C for 20 min. Formation of APNH was observed with CYP1A1, CYP1A2, CYP1B1, CYP2B6, CYP2D6, CYP2E1 and CYP3A4, but not with CYP2A6, CYP2C9 and CYP2C19. The amounts of APNH from norharman and aniline were 33 ng for CYP1A1, 15 ng for CYP3A4, 7 ng for CYP2D6, 6 ng for CYP1A2 and 5 ng for CYP2B6. APNH formation in the presence of CYP1B1 and CYP2E1 was very low at around one fiftieth of that with CYP3A4. When CYP selective chemical inhibitors, such as furafylline for CYP1A2 and ketoconazole for CYP3A4, were added to the reaction mixture of norharman, aniline and human microsomes, formation of APNH was decreased to 14 and 16% of the control level, respectively. Moreover, human lung microsomes also showed the activity of APNH formation from norharman and aniline, albeit at only one hundredth of that with liver microsomes. In general, content in human liver microsomes is rather high for CYP3A4 and CYP1A2 but relatively low for CYP2D6 and CYP2B6, at about 30, 10, 1.5% and less than 1% of the total CYP, respectively. Although CYP1A1 showed the highest APNH formation activity, its expression in human liver is reported to be below the level of detection. Based on these observations, it is suggested that the practical major contributors to the formation of APNH from norharman and aniline are CYP3A4 and CYP1A2, the responsible reactions mainly occurring in the liver.
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Keywords: Norharman; Aniline; Aminophenylnorharman (APNH); Cytochrome P-450

1. Introduction

Norharman (9*H*-pyrido[3,4-*b*]indole), β-carboline compound, is not mutagenic to *Salmonella typhimurium* strains, either with or without S9 mix. However, it becomes mutagenic to *S. typhimurium* TA98 and YG1024 with S9 mix in the presence of non-mutagenic aromatic amines, such as aniline

Abbreviations: APNH, aminophenylnorharman [9-(4′-aminophenyl)-9*H*-pyrido[3,4-*b*]indole]; CYP, cytochrome P-450; HCAs, heterocyclic amines; MeIQx, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline; PHA, phenylhydroxylamine; thiotepa, triethylenethiophosphoramide

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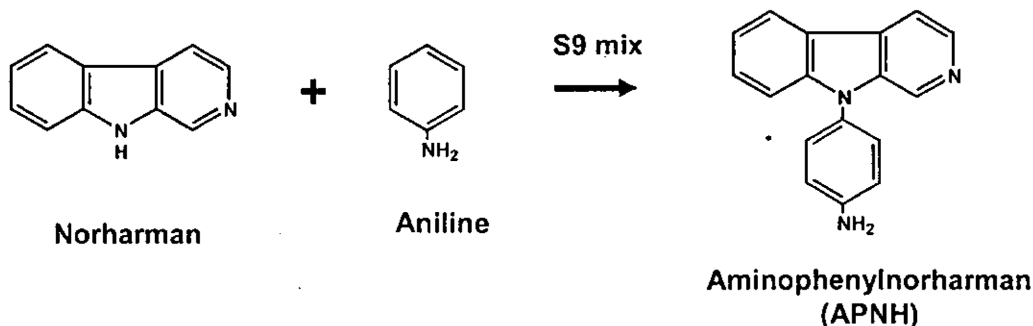

Fig. 1. Formation of mutagenic aminophenylnorharman by a reaction of norharman with aniline in the presence of S9 mix.

and *o*-toluidine [1–3]. We have studied the mechanism of the co-mutagenic action of norharman with aniline, and the appearance of mutagenicity derived from a mixture of these two in the presence of S9 mix was found to be due to the formation of a mutagenic compound, 9-(4′-aminophenyl)-9*H*-pyrido[3,4-*b*]indole (aminophenylnorharman, APNH), as shown in Fig. 1 [4,5]. APNH is thought to be metabolically converted to hydroxyamino derivative and further activated to form esters by acetyltransferase, then covalently binding to DNA bases. The major APNH–DNA adduct has been concluded to be $N^{4'}$-(2′-deoxyguanosin-8-yl)-9-(4′-aminophenyl)-9*H*-pyrido[3,4-*b*]indole (dG-C8-APNH) [6]. Mutagenic activity of APNH in *S. typhimurium* strains was found to be comparable to those of 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole, two carcinogenic heterocyclic amines (HCAs) formed in cooked foods [4,7].

Norharman is produced in the pyrolysis of tryptophan [8] and is reported to be present in cigarette smoke condensate at levels of 900–8990 ng/cigarette and in various kinds of cooked foods at levels of 2.4–795 ng/g foods [9]. These levels are much higher than those of mutagenic/carcinogenic HCAs, including 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine and MeIQx. Similarly, aniline has also been reported to be present in cigarette smoke condensate and some kinds of vegetables [10,11]. Moreover, both norharman and aniline have been detected in human urine and breast milk samples [12–15]. Thus, it is assumed that humans are simultaneously exposed to norharman and aniline in everyday life. We have already reported that when norharman and aniline were administrated to rats by gavage, APNH was detected in the urine. In addition, a microsomal fraction from human liver was demonstrated to have the capacity to produce APNH from norharman and aniline [16].

From these observations, formation of APNH in the human body might be expected. Our previous report indicated that cytochrome P-450s (CYPs) could be involved in the APNH formation from norharman and aniline in the presence of a liver microsome fraction, because reduction of APNH formation activity was clear on addition of the CYP inhibitor, SKF-525A. However, it has hitherto not been elucidated what types of CYP isoenzyme(s) might be involved in the reaction. CYPs consist of a large superfamily of proteins including four families, CYP1, CYP2, CYP3 and CYP4, that appear to be primarily involved in metabolism of xenobiotics.

To clarify the reaction mechanisms of norharman and aniline, it is very important to determine what CYPs are involved in the formation of APNH. In the present study, norharman and aniline were incubated in the presence of various recombinant human CYPs and the formation of APNH was analyzed by HPLC. Based on the data obtained, possible formation of APNH in the human body is also discussed.

2. Materials and methods

2.1. Materials

Norharman hydrochloride and aniline were purchased from Katsura Chemical (Tokyo, Japan) and Wako Pure Chemical Industries (Osaka, Japan), respectively. Ketoconazole and furafylline were

obtained from Ultrafine Chemicals (Manchester, UK). Triethylenethiophosphoramide (thiotepa) was obtained from Acros Organics (New Jersey, USA). Rat S9 (20 mg protein/ml), prepared from the livers of male Sprague-Dawley rats treated with both phenobarbital and β-naphthoflavone, was purchased from Kikkoman Co. Ltd. (Noda, Japan). Microsomal fractions from human liver and lung were obtained from Gentest (Woburn, MA, USA) and XenoTech LLC (Kansas, USA), respectively. Microsomes from baculovirus-infected insect cells expressing human CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4 were purchased from Gentest. All recombinant CYPs were coexpressed with NADPH-P450 oxidoreductase. Additionally, recombinant CYP2E1 was expressed with cytochrome b_5. Microsomal fractions obtained from insect cells, expressing only the vector, were used as a control. All other chemicals employed were analytical grade.

2.2. Analysis of APNH formation

Norharman (8 mg) and aniline (4 mg) dissolved in 8 ml of water were incubated with 20 ml of enzyme solution containing individual recombinant human CYPs (CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4) for 20 min at 37 °C, as described previously [4,17,18]. The reaction mixture consisted of 8 μM $MgCl_2$, 33 μM KCl, 4 μM NADPH, 5 μM D-glucose 6-phosphate, 100 μM sodium phosphate buffer (pH 7.4) and 2 nmol of each recombinant human CYPs. In some experiments, human liver or lung microsomes (40 or 20 mg protein, respectively) were used instead of recombinant human CYPs. For inhibition studies, the mixtures of norharman and aniline with human liver microsome described above were combined with 10 μM furafylline [19], 75 μM thiotepa [20] or 1 μM ketoconazole [21], known to selectively inhibit the activities of CYP1A2, CYP2B6 and CYP3A4, respectively. The concentrations of the CYP selective chemical inhibitors employed in the present study were similar to those reported previously [19–21].

APNH, produced by the reaction of norharman with aniline in the presence of each enzyme, was analyzed by HPLC with the procedure detailed previously [4]. Briefly, after removing proteins from the reaction mixture, the supernatant was evaporated. Then, the residue dissolved in 1 ml of 50% methanol was separated by HPLC as follows. The test material was applied to an analytical grade TSK gel ODS-80Ts column (5 μM particle size, 4.6 mm × 250 mm; Tosoh, Tokyo, Japan) and eluted at a flow rate of 1 ml/min with following gradient system: 0–30 min, 15% acetonitrile in 25 mM phosphate buffer (pH 2.0); 30–60 min, a linear gradient of 15–50% acetonitrile in 25 mM phosphate buffer (pH 2.0). The fractions with retention times of 32–38 min, corresponding to those of authentic APNH, were collected. These were combined, evaporated and the residue was dissolved in 1 ml of 50% methanol. An aliquot of the solution was injected into the same column and a mobile phase of 18% acetonitrile in 25 mM phosphate buffer (pH 2.0) was pumped in isocratically. The samples with retention times corresponding to authentic APNH, 18–22 min, were collected and further purified using the same column with a mobile phase of 45% acetonitrile in water with 0.1% of diethylamine adjusted to pH 6.0 with acetic acid. Finally, a single UV absorption peak was obtained with the same elution position of authentic APNH at retention times of 14–15 min. The compound in the peak fraction showed mutagenic activity to *S. typhimurium* YG1024 in the presence of S9 mix and its UV spectrum was consistent with authentic APNH. Therefore, we concluded this mutagen to be APNH.

All the above HPLC procedures were performed at the ambient temperature at a flow rate of 1 ml/min. Monitoring of UV absorbance of the eluate was conducted at 254 nm and UV absorbance spectra were measured with an SPD-M10Avp photodiode array detector (Shimadzu, Kyoto, Japan). Amounts of APNH were calculated as average values with data from two assays.

3. Results and discussion

Norharman and aniline were incubated with individual recombinant human CYPs at 37 °C for 20 min, and the amounts of APNH were analyzed by HPLC on an analytical ODS column according to the procedure described in Section 2. To confirm the structure of the mutagen isolated from the reaction mixture of norharman, aniline and recombinant human CYPs,

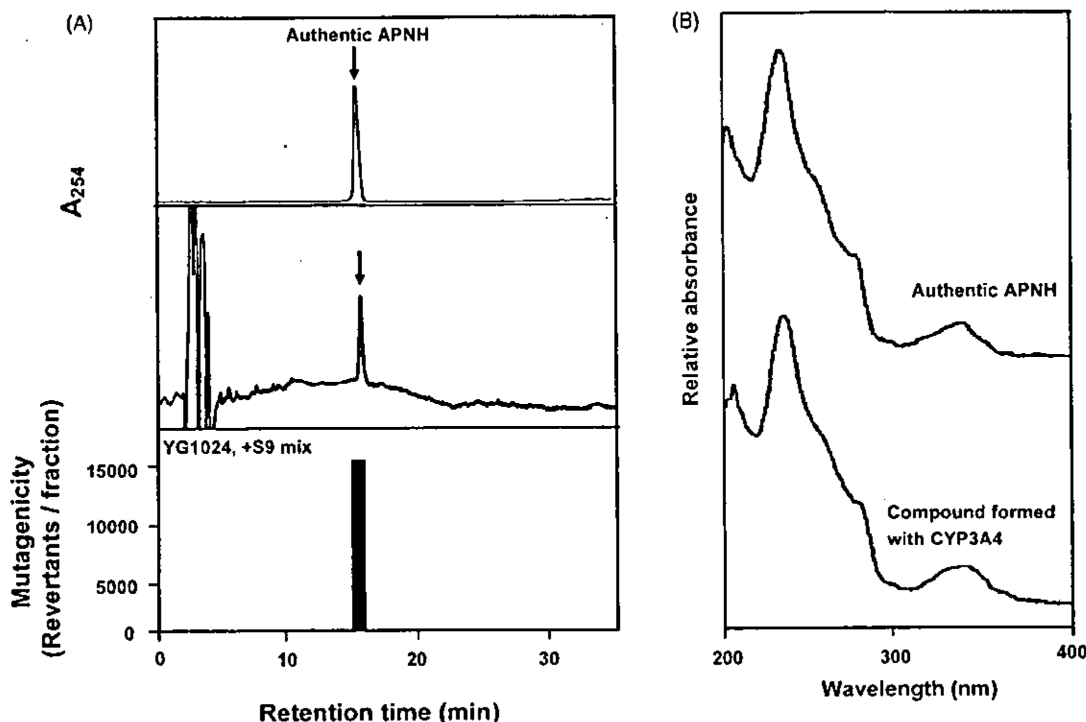

Fig. 2. HPLC pattern and UV absorption spectrum of authentic APNH and a reaction mixture with norharman and aniline in the presence of CYP3A4. (A) The test material was separated on an analytical ODS-80Ts column with a mobile phase of 45% acetonitrile in water with 0.1% diethylamine adjusted to pH 6.0 with acetic acid. The UV absorbance of the elute was monitored at 254 nm and mutagenicity of the fraction was examined in *S. typhimurium* YG1024 with S9 mix. The UV absorption peak showing mutagenicity is indicated by an arrow. (B) UV absorption spectra measured with a photodiode array detector. The upper spectrum is for authentic APNH and the lower one for the compound formed with CYP3A4.

mutagenic activities and UV absorbance spectrum were analyzed. When norharman and aniline were incubated with CYP3A4, a single UV absorption peak showing mutagenicity was observed at the same elution position as for authentic APNH (Fig. 2A). Since the UV absorption pattern was identical with that for authentic APNH, the mutagen produced by the reaction of norharman and aniline in the presence of CYP3A4 was confirmed to be APNH (Fig. 2B). Mutagenicities and amounts of APNH in the reaction mixture of norharman and aniline with each recombinant human CYP are summarized in Table 1. With CYP1A1, CYP1A2, CYP1B1, CYP2B6, CYP2D6, CYP2E1 and CYP3A4 as the enzymes, mutagenic activity was observed for the fraction corresponding to the authentic APNH elution position. Among the isoforms, CYP1A1 generated the highest mutagenicity, followed by CYP3A4, CYP2D6, CYP1A2 and CYP2B6. Only weak mutagenic activity was noted with CYP1B1 and CYP2E1. As shown in Table 1, a good correlation was observed between mutagenicity and the amount of APNH. When norharman (8 mg) and aniline (4 mg) were incubated with 2 nmol of each CYP1A1, CYP1A2, CYP2B6, CYP2D6 or CYP3A4, APNH was produced at levels of 5–33 ng. Although weak mutagenicity was observed with CYP1B1 and CYP2E1, APNH was under the detection limit, and the amounts would be less than 1 ng, if present. CYP2A6, CYP2C9 and CYP2C19 did not generate any mutagenic activity and presumably did not catalyze formation of APNH from norharman and aniline.

The contents of the four positive CYP species in human liver have been reported to be 110–360 pmol for CYP3A4, 27–56 pmol for CYP1A2 and 0.4–9 pmol for CYP2B6 and CYP2D6 per mg protein, respectively [22–25]. On the other hand, CYP1A1 is considered

Table 1
Amounts of APNH and mutagenicity of reaction mixtures of norharman and aniline in the presence of CYPs

CYP species	Mutagenicity per reaction mixture	Amount of APNH per reaction mixture (ng)
1A1	120000	33
1A2	21000	6
1B1	1000	ND
2A6	0	ND
2B6	19000	5
2C9	0	ND
2C19	0	ND
2D6	26000	7
2E1	900	ND
3A4	54000	15
Control[a]	0	ND

ND: not detected (<1 ng per reaction mixture). A reaction mixture containing 8 mg norharman, 4 mg aniline and 2 nmol of each CYPs was incubated at 37 °C for 20 min, and then the amount of APNH was analyzed by HPLC. The mutagenicity of the fraction containing APNH was examined in *S. typhimurium* YG1024 with S9 mix.

[a] Microsomal fraction obtained from insects cells expressing only the vector.

Table 2
Inhibition of APNH formation by selective CYP inhibitors

	Amount of APNH (ng)	Inhibition rate (%)
Human liver microsome	881	
Human liver microsome + furafylline (10 μM)	122	86
Human liver microsome + ketoconazole (1 μM)	143	84

A reaction mixture containing 8 mg norharman, 4 mg aniline and each selective CYP inhibitor in the presence of human liver microsome was incubated at 37 °C for 20 min, and the amount of APNH was analyzed by HPLC. Furafylline and ketoconazole selectively inhibit the activities of CYP1A2 and CYP3A4, respectively. The experiments were performed in duplicate, and the data expressed are mean values.

to be an extrahepatic enzyme, because its mRNA and protein have been detected in the lung, lymphocyte and placenta, but not in most human liver [26–28]. Only a few reports have described expression levels of CYP1A1 in human liver [25,29,30]. In general, CYP1A1 can be inducible with various substances, including benzo[*a*]pyrene, polychlorinated biphenyl and indole-3-carbinol via the aryl hydrocarbon receptor signaling pathway, whereas the expression levels of human liver microsomes are as low as negligible. From the APNH formation activity of each CYPs and their expression levels of human liver, it is suggested that CYP3A4 and CYP1A2 might mainly contribute to the reaction of norharman and aniline. Therefore, we examined the effect of selective chemical inhibitors, furafylline (CYP1A2) and ketoconazole (CYP3A4), on the formation of APNH in the presence of human liver microsome to clarify their involvement. Two or three concentrations of each selective inhibitor were examined in the present study, and the most effective doses for prevention of APNH formation are presented in Table 2. Furafylline and ketoconazole exhibited inhibition in the presence of human liver microsomes to 14 and 16% of the control level, respectively. In contrast, CYP2B6 selective inhibitor, thiotepa (75 μM), demonstrated weak inhibition of APNH formation (around 75% of the control level). Moreover, CYP2D6 showed similar level of APNH formation activity and content in human liver as those of CYP2B6. Thus, CYP2B6 and CYP2D6 may not be involved in the reaction of norharman and aniline as a major contributor.

As mentioned above, CYP3A4 is the most abundant CYP isoenzyme in the human liver, and APNH formation was well inhibited by its specific inhibitor, ketoconazole. Therefore it is suggested that CYP3A4 may be one major contributor to production of APNH in human liver microsome. Similarly, the CYP1A2 inhibitor, furafylline, exerted strong suppressive effect. Although the APNH formation was on half that with CYP3A4, the CYP1A2 level presented in human liver is sufficient for a role in vivo.

CYP1A1 may be found in extrahepatic tissues, for example, in the lungs of smokers and in the placenta [26–28] and CYP2B6 and CYP3A4 proteins can also be expressed in human lung. Moreover, norharman and aniline are both present in cigarette smoke. Therefore, we examined the ability of human lung microsomes to generate APNH when incubated with the two precursors. Mutagenicity was seen at the same elution position as for authentic APNH on HPLC, but the level was only one hundredth of that with human liver microsomes.

The mechanisms of the APNH formation from norharman and aniline have yet to be clarified in detail, but Guengerich has suggested the possibility that

the two amines are brought together in a P450 complex so an *ipso* attack on the aniline can occur [31]. On the other hand, it has been reported that a mixture of norharman and phenylhydroxylamine (PHA) which is an *N*-hydroxy derivative of aniline, in the presence of S9 mix, yield three times higher mutagenicity than the mixture of norharman and aniline [3]. Moreover, we confirmed that APNH was formed in the reaction mixture of norharman and PHA with S9 mix. Thus, another possible mechanism of formation of APNH can be postulated: first, aniline is oxidized to form PHA by CYPs, which might then be converted to a phenyl nitrenium ion by further enzymatic reactions. In fact, it is known that CYP1A1, CYP1A2 and CYP3A4 are mostly involved in metabolic activation of HCAs through *N*-oxidation [32,33]. As reactive intermediates, carbocations derived from aryl nitrenium ions at the C-4 position might be produced, and could couple at the N-9 position of norharman to form APNH. It has been documented that 7-hydroxynorharman become detectable in the urine of rats administrated norharman [34], suggests that the compound might be also oxidized by oxidative enzymes such as CYPs. At present, data to unequivocally identify the reaction mechanisms of oxidative norharman and aniline are lacking, but if the N-9 position of norharman is to be oxidized to form N9-hydroxynorharman, this might be lead to generation of a nitrenium ion by further enzymatic reactions and this could couple at the C-4 position of aniline to form APNH. Further studies are warranted for clarification.

As mentioned above, norharman and aniline are widely distributed in our environment, for example in cooked foods, some vegetables and cigarette smoke condensate, and it is likely that APNH may be produced in our bodies [9,10]. To determine whether this is actually the case, we are now looking for APNH in 24 h human urine samples.

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COMBINED EFFECTS OF CYCLOOXYGENASE-1 AND CYCLOOXYGENASE-2 SELECTIVE INHIBITORS ON INTESTINAL TUMORIGENESIS IN *ADENOMATOUS POLYPOSIS COLI* GENE KNOCKOUT MICE

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As with cyclooxygenase (COX)-2, genetic disruption of *COX-1* gene or pharmacologic inhibition of its activity has been shown to decrease the number of intestinal polyps in *Apc* gene-deficient mice. The present study was designed to investigate the combined effects of COX-1 and COX-2 selective inhibitors on spontaneous polyp formation in *APC1309* female mice. The animals were treated with 300 or 600 ppm mofezolac (a COX-1 selective inhibitor) alone, 200 or 400 ppm nimesulide (a COX-2 selective inhibitor) alone, 300 ppm mofezolac plus 200 ppm nimesulide, 600 ppm mofezolac plus 400 ppm nimesulide, or 10 ppm indomethacin (a dual-COX inhibitor) in the diet from 7 weeks of age for 4 weeks. Percentage inhibition of polyp area in the intestine was 17% with 600 ppm mofezolac alone and 25% with 400 ppm nimesulide alone, their sum of 42% being almost equal to the 37% observed for the combination treatment. Administration of 300 ppm mofezolac plus 200 ppm nimesulide also significantly decreased polyp area in the intestine by 30%. Moreover, the numbers of polyps more than 2.5 mm in diameter were markedly decreased by combined treatment of both COX inhibitors. With 10 ppm indomethacin, the dual inhibitor, polyp area was also clearly reduced by 46%. Our results indicate that COX-1 and COX-2 may to some extent contribute to polyp formation independently and inhibitor combination treatment thus has particular potential for chemoprevention of colon carcinogenesis.
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Key words: *Apc knockout mice; COX-1 selective inhibitor; COX-2 selective inhibitor; intestinal tumorigenesis; combined effect*

Long-term use of aspirin, a nonsteroidal antiinflammatory drug (NSAID), has been found in the epidemiologic studies to result in reduction in mortality rate from colorectal cancer.[1,2] Furthermore, treatment with NSAIDs suppresses the development of chemical carcinogen-induced colon cancers in experimental animals.[3,4] Similarly, decrease has been achieved with such agents regarding intestinal polyps in patients with familial adenomatous polyposis (FAP)[5–7] and in an animal model of the disease, mutant mice with a truncated *adenomatous polyposis coli* (*Apc*) gene.[8] NSAIDs block arachidonic acid metabolism by inhibiting cyclooxygenase (COX) activity and thus reducing levels of prostaglandins, which affect cell proliferation, tumor growth, apoptosis and immune responsiveness. Two enzyme isoforms of COX are known, referred to as COX-1 and COX-2. COX-1 is constitutively expressed in most tissues and plays a role in various physiologic functions, while COX-2 is transiently inducible by stimuli such as cytokines, growth factors and hormones and contributes to inflammation.[9] By using COX-2 selective inhibitors or mutant mice featuring disruption of the gene encoding this enzyme, relevance to carcinogenesis in various organs, including the colon, has been shown.[10–13]

Possible involvement of COX-1 in colon carcinogenesis has also been reported. Genetic disruption of the *COX-1* gene decreased the number of intestinal polyps in Min mice by around 80%.[14] In these animals, COX-1 protein is detectable in the lamina propria and inner muscular layer of the normal small intestine and intestinal polyps, while COX-2 is found only in the lamina propria of polyps.[14] Moreover, we recently demonstrated that mofezolac, a COX-1 selective inhibitor, suppressed the development of azoxymethane (AOM)-induced colonic aberrant crypt foci (ACFs), putative preneoplastic lesions in F344 rats and intestinal polyp development in mice with a truncated *Apc* gene at codon 1309 (*APC1309* mice).[15] It should be noted that prostaglandin E_2 (PGE_2) levels were increased in intestinal polyps in Min mice compared with surrounding normal tissue, and genetic disruption of the *COX-1* gene, as well as the *COX-2* gene, indicated that both isoforms contributed to PGE_2 production.[14] PGE_2 exerts its biologic actions through binding to the 4 specific membrane receptor subtypes EP_1, EP_2, EP_3 and EP_4.[16,17] With genetic and/or pharmacologic approaches, it has been revealed that PGE_2 is involved in intestinal carcinogenesis through its binding to the PGE_2 receptor subtypes EP_1, EP_2 and EP_4.[18–20] Thus, the available evidence points to significant roles for PGE_2, produced by COX-1 and COX-2, in colon carcinogenesis.

In FAP patients, conventional NSAIDs, indomethacin[7] or sulindac,[5] which inhibit both COX-1 and COX-2, have been found to reduce the number of intestinal polyps more effectively than the COX-2 selective inhibitors, celecoxib[21] and nimesulide,[22] suggesting possible contributions of both COX-1 and COX-2 to human intestinal tumorigenesis. Thus, we here postulate that a combination of COX-1 and COX-2 inhibitors should decrease the number of intestinal polyps more effectively than either agent alone, assuming independent contributions to polyp formation in *Apc* gene-deficient mice. To test this possibility, combined effects of mofezolac, a COX-1 selective inhibitor, and nimesulide, a COX-2 selective inhibitor, on spontaneous polyp formation in *APC1309* mice were examined.

Abbreviations: ACFs, aberrant crypt foci; AOM, azoxymethane; *Apc*, *adenomatous polyposis coli*; COX, cyclooxygenase; FAP, familial adenomatous polyposis; mofezolac, [3,4-di(4-methoxyphenyl)-5-isoxazolyl]acetic acid; nimesulide, 4-nitro-2-phenoxymethanesulfonanilide; NSAID, nonsteroidal antiinflammatory drug; PGE_2, prostaglandin E_2.

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MATERIAL AND METHODS

Animals and chemicals

Progeny of *APC1309* mice, produced by a gene knockout method and bred by artificial insemination, were genotyped by the allele-specific polymerase chain reaction using tail tips.[15,23] Mice at 7 weeks of age were randomized into experimental and control groups. The animals were housed 2 or 3 to a plastic cage in a holding room controlled at 24 ± 2°C and at 55% relative humidity with a 12/12-hr light-dark cycle. Water and basal diet (CE-2, Clea Japan, Tokyo, Japan) with or without test chemicals were provided *ad libitum* during the experiments. The animals were weighed weekly throughout the experiment. Mofezolac, [3,4-di(4-methoxyphenyl)-5-isoxazolyl]acetic acid (Mitsubishi Pharma, Chikujo-gun, Fukuoka, Japan), a COX-1 selective inhibitor,[24] has been clinically used to control acute pain and inflammation from operation, injury, or odontectomy. Nimesulide, 4-nitro-2-phenoxymethanesulfonanilide, a COX-2 selective inhibitor, was kindly provided by Helsinn Healthcare (Pazzallo-Lugano, Switzerland). Their structures are shown in Figure 1. Experimental diets were prepared weekly by thoroughly mixing each compound into the basal diet. The chemicals were confirmed to be stable under the experimental conditions used in the present study. The experimental protocol was in accordance with the guideline for animal experiments of the National Cancer Center.

Experimental protocol

Just prior to starting treatment, 7 *APC1309* heterozygous female mice (group 1) at 7 weeks of age were sacrificed to obtain predosing data for intestinal polyps. Other *APC1309* female mice starting at 7 weeks of age were fed a control diet (group 2) or experimental diets containing 300 ppm or 600 ppm mofezolac (groups 3 and 4, respectively), 200 ppm or 400 ppm nimesulide (groups 5 and 6, respectively), 300 ppm mofezolac plus 200 ppm nimesulide (group 7), 600 ppm mofezolac plus 400 ppm nimesulide (group 8), or 10 ppm indomethacin (group 9) throughout the experiment. For comparison, wild-type animals received the control diet (group 10), 600 ppm mofezolac (group 11), 400 ppm nimesulide (group 12), 600 ppm mofezolac plus 400 ppm nimesulide (group 13), or 10 ppm indomethacin (group 14). Six hundred ppm mofezolac and 400 ppm nimesulide were selected because they were previously reported to suppress polyp development in the mice.[15] We also set half-dose levels to avoid possible additive side effects in the combination treatment groups. Ten ppm indomethacin was selected as the maximum tolerated dose level based on preliminary study in our laboratory (data not shown). The numbers of animals per group were 7 (group 1) and 8 (groups 2–9) for the knockout animals and 5 for wild-type animals (groups 10–14). After 4 weeks of treatment, complete necropsies were performed on all animals, and the liver, kidneys and spleen were weighed on sacrifice 1 hr after being injected i.p. with 50 mg per kg body weight bromodeoxyuridine (BrdU; Sigma Chemical, St. Louis, MO). The entire intestinal tract was resected, filled with 10% neutral buffered formalin and divided into the small intestine, cecum and colon. The small intestine was divided into the duodenum (4 cm in length; proximal) and the proximal (middle) and distal halves of the remainder (distal). These segments were opened longitudinally and fixed flat between sheets of filter paper in 10% neutral buffered formalin. The numbers and sizes of polyps as well as their distribution in the intestine were determined under ×5 magnification with a stereoscopic microscope, detectable polyps being more than 0.2 mm in diameter.[15] Measurement of polyp size was made using an eyepiece micrometer. After counting polyps, the small intestines were embedded in paraffin and sections were submitted to immunohistochemistry using an anti-BrdU antibody and a Vectastain ABC kit (Vector Laboratories, Burlingame, CA). For determination of BrdU labeling indexes of the crypt epithelium, 21 well-oriented crypts, in which the base, lumen and top could be clearly seen, were counted for each of 5 animals per group, 7 crypts each for the proximal, middle and distal small intestine. The percentage of labeled cells (labeling index) was determined for the whole crypt by calculating the labeled cells per total number of cells × 100. In the case of BrdU labeling indexes of the polyps, 3 polyps were counted for each of 5 animals per group, 1 polyp each for the proximal, middle and distal small intestine. The percentage of labeled cells (labeling index) was determined for approximately 460 cells per polyp on average by calculating the labeled cells per total number of cells × 100. Paraffin sections of intestinal polyps excised from mice of groups 2–9 and the stomachs and small intestines excised from mice of groups 2–14 were also examined microscopically following routine processing and H&E staining.

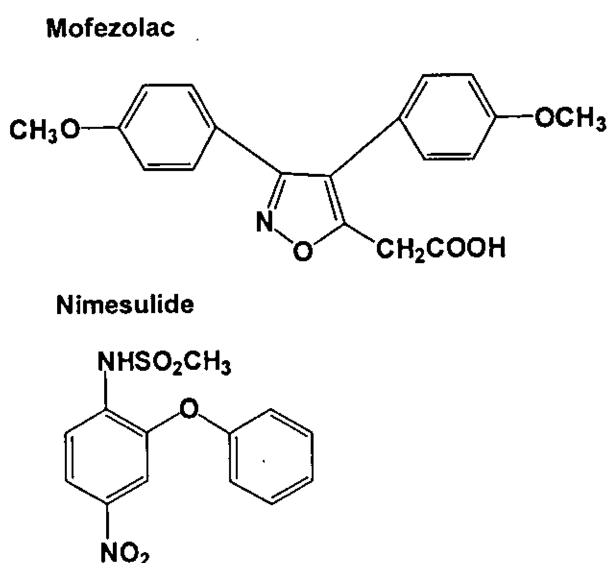

FIGURE 1 – Chemical structures of mofezolac and nimesulide.

Statistical analysis

Statistical analysis was performed using Welch's *t*-test. Each of the experimental diet groups (groups 3–9 and 11–14) was compared with the respective basal diet groups (groups 2 and 10) and the predose sacrifice group (group 1) was compared with the basal diet group (group 2). In addition, each of single COX-1 or COX-2 inhibitor treatment groups was compared with the respective combined inhibitor treatment groups (groups 3 and 5 compared with group 7, groups 4 and 6 compared with group 8). Results were considered statistically significant when $p < 0.05$.

RESULTS

Body weights and food intake were not influenced by treatment with COX-1 and/or COX-2 selective inhibitors, independent of the genotype. No adverse effects were observed regarding necropsy findings and organ weights. Intestinal polyps were detected in all *APC1309* mice but not in any of the wild-type animals. Histopathologic analysis revealed no differences in the morphology of polyps, identified as adenomas, between the *APC1309* mouse groups fed the basal diet and those given test chemicals. Moreover, there were no treatment-related lesions observed in the glandular stomachs or small intestines.

In order to analyze the effects of combination treatments of COX-1 and COX-2 inhibitors on polyp growth, the area of each polyp was calculated using the formula: area = (long diameter × short diameter × π). Table I shows values for the mean polyp areas for the different intestinal segments in each group. The mean polyp areas for the whole intestine in the 300 ppm mofezolac alone (group 3), 200 ppm nimesulide alone (group 5) and the combination treatment (group 7) groups were 106%, 85% and 70% of the control (group 2) value (mean, 7.9 mm^2), respectively. Although

TABLE I – MEAN AREAS OF INTESTINAL POLYPS AFTER 4 WEEKS OF TREATMENT WITH MOFEZOLAC AND/OR NIMESULIDE OR INDOMETHACIN IN *APC1309* MICE

Group number	Treatment[1]	Mean area of intestinal polyps per mouse (mm^2)[2]				
		Small intestine			Colon	Whole intestine[3]
		Proximal	Middle	Distal		
1	Predose sacrifice	4.6 ± 2.9[4]	4.2 ± 1.2[4]	3.1 ± 0.7[5]	0.6 ± 0.6	3.4 ± 0.5[4]
2	Basal diet	15.6 ± 7.3	8.7 ± 3.3	4.7 ± 1.8	1.3 ± 3.0	7.9 ± 2.1 (100%)
3	Mofezolac 300 ppm	13.0 ± 5.2	10.2 ± 2.9[7]	4.1 ± 1.6	1.7 ± 2.1	8.5 ± 2.6[5] (106%)
4	Mofezolac 600 ppm	14.7 ± 7.5[6]	8.2 ± 2.7	2.5 ± 0.7[5]	0.7 ± 1.0	6.6 ± 2.2 (83%)
5	Nimesulide 200 ppm	10.8 ± 3.7	7.5 ± 2.8	4.0 ± 0.6	0.4 ± 0.3	6.7 ± 2.0 (85%)
6	Nimesulide 400 ppm	10.4 ± 2.4[6]	6.9 ± 2.0	3.6 ± 1.4	0.9 ± 1.0	5.9 ± 1.4[5] (75%)
7	Mofezolac 300 ppm + nimesulide 200 ppm	10.8 ± 4.5	5.7 ± 1.6[5]	3.5 ± 1.1	1.4 ± 1.3	5.6 ± 1.6[5] (70%)
8	Mofezolac 600 ppm + nimesulide 400 ppm	6.9 ± 3.1[5]	6.3 ± 1.8	2.8 ± 0.9[5]	1.6 ± 2.1	5.0 ± 1.4[4] (63%)
9	Indomethacin 10 ppm	7.7 ± 3.3[5]	5.2 ± 2.0[5]	2.3 ± 0.7[4]	0.4 ± 0.4	4.3 ± 1.5[4] (54%)

Parentheses: group means expressed as percentage compared with the control group.–[1]Mice were fed the basal diet or diets containing compounds starting at 7 weeks of age for 4 weeks. Animals in group 1 were sacrificed at the commencement. The numbers of animals per group were 7 for group 1 and 8 for groups 2 to 9.–[2]Data are mean ± SD values.–[3]Whole intestine means both small intestine and colon.–[4]$p < 0.01$ *vs.* the basal diet group (group 2).–[5]$p < 0.05$ *vs.* basal diet group (group 2).–[6]$p < 0.05$ *vs.* each combination group (group 7 or 8).–[7]$p < 0.01$ *vs.* each combination group (group 7 or 8).

300 ppm mofezolac alone showed no inhibition and 200 ppm nimesulide alone showed 15% inhibition, the combination caused 30% inhibition. The mean polyp areas for the whole intestine in the 600 ppm mofezolac alone (group 4), 400 ppm nimesulide alone (group 6) and the combination treatment (group 8) groups were 83%, 75% and 63% of the control value, respectively. The sum (42%) for the 2 agents given alone (17% + 25%) was almost equal to that with their combination treatment (37%). Suppressive effects with the combination of these agents, as well as indomethacin, were generally consistent throughout the small intestinal segments, but not in the colon. In the proximal small intestine, where the mean polyp area in the controls was largest, the value for the combination group (600 ppm mofezolac plus 400 ppm nimesulide) was significantly smaller than with the single treatment groups at the same dose levels. After treatment with 10 ppm indomethacin, the mean polyp area was 54% of the control value. Figure 2 summarizes data for percentage inhibition of polyp areas in the intestine for groups 3–9.

Data for the numbers of polyps classified by diameter are shown in Table II. When compared with predose mice (group 1), the numbers of polyps measuring 1.5 mm or more in diameter were significantly increased in the control mice (group 2) fed basal diet for 4 weeks. Single treatment with indomethacin and combination treatment with mofezolac (300 and 600 ppm) and nimesulide (200 and 400 ppm) for 4 weeks decreased the numbers of polyps measuring 2.5 mm or more in diameter, with increase in those measuring less than 1.5 mm, compared with value of control mice (group 2). Statistical significance was observed except for the number of polyps measuring 3.5 mm or more in the combination group of 300 ppm mofezolac and 200 ppm nimesulide. The 10 ppm indomethacin clearly retarded the growth of intestinal polyps, and similar suppressing effects were obtained with the combination of mofezolac and nimesulide.

There were no clear effects of any treatment on the numbers of intestinal polyps; the values were 39.0 ± 7.4 (mean ± SD) per mouse for the predose group, 57.8 ± 21.3 for the controls, 57.8 ± 14.6 for 300 ppm mofezolac alone, 41.8 ± 11.3 for 600 ppm mofezolac alone, 55.3 ± 13.6 for 200 ppm nimesulide alone, 69.6 ± 27.6 for 400 ppm nimesulide alone, 65.6 ± 20.5 for the combination of 300 ppm mofezolac and 200 ppm nimesulide, 55.0 ± 11.7 for the combination of 600 ppm mofezolac and 400 ppm nimesulide and 50.8 ± 13.5 for 10 ppm indomethacin alone. Moreover, there were no significant effects of any treatment on total polyp area.

BrdU labeling indexes of the crypt epithelium and the polyps are shown in Table III. Among *APC1309* mice, all labeling indexes of the crypt epithelium and the polyps of treated groups (groups 3–9) were lower than that of the basal diet group (group 2) and statistical significances were observed in all groups except for the labeling index of the crypt epithelium in the group treated with 300 ppm mofezolac. On the other hand, among wild-type mice, no treatment-related changes were detected and the labeling indexes of the crypt epithelium of all groups were significantly lower than that of the basal diet group of knockout mice (group 2).

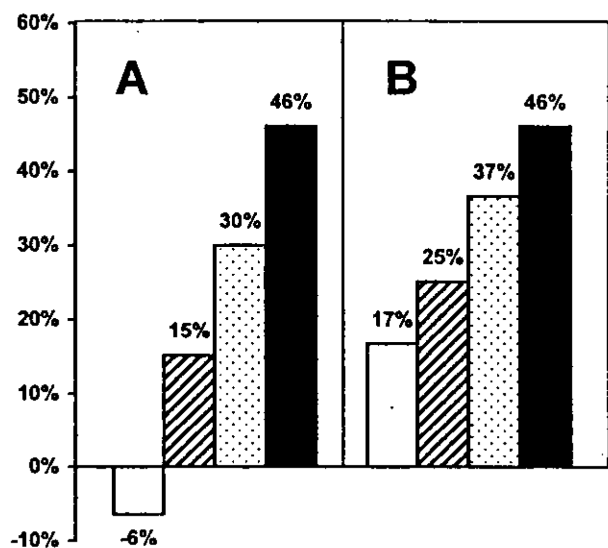

FIGURE 2 – Combined effects of mofezolac and nimesulide on mean areas of intestinal polyps. Mice were fed the basal diet or diets containing compounds starting at 7 weeks of age for 4 weeks. Treatment effects are expressed as percentage inhibition compared with the control value (group 2). (*a*) Bars showing 300 ppm mofezolac alone (open bar), 200 ppm nimesulide alone (hatched bar), 300 ppm mofezolac plus 200 ppm nimesulide (dotted bar) and 10 ppm indomethacin (closed bar). (*b*) Bars showing 600 ppm mofezolac alone (open bar), 400 ppm nimesulide alone (hatched bar), 600 ppm mofezolac plus 400 ppm nimesulide (dotted bar) and 10 ppm indomethacin (closed bar).

DISCUSSION

The present study was conducted to assess how polyp development is modified by a combination of COX-1 and COX-2 inhibitors compared with effects of COX selective inhibitors alone. Analyses of polyp diameter distribution and mean polyp area revealed that combination treatment with COX-1 and COX-2 selective inhibitors more effectively suppressed polyp growth than either of the single treatments alone. While combination effects

TABLE II – SIZE DISTRIBUTIONS OF INTESTINAL POLYPS AFTER 4 WEEKS OF TREATMENT WITH MOFEZOLAC AND/OR NIMESULIDE OR INDOMETHACIN IN *APC1309* MICE

Group number	Treatment[1]	Longer diameter of polyps (mm)[2]				
		≥ 0.2 and < 0.5	≥ 0.5 and < 1.5	≥ 1.5 and < 2.5	≥ 2.5 and < 3.5	≥ 3.5
1	Predose sacrifice	4.4 ± 2.1	26.9 ± 5.1	6.1 ± 2.6[3]	0.6 ± 0.5[3]	0 ± 0[4]
2	Basal diet	7.4 ± 3.2	21.8 ± 8.0	19.1 ± 10.0	7.6 ± 4.9	2.0 ± 1.9
3	Mofezolac 300 ppm	4.0 ± 2.2[4]	25.5 ± 6.1	17.8 ± 5.2	7.5 ± 6.8	2.1 ± 2.7
4	Mofezolac 600 ppm	4.4 ± 1.3[4]	21.1 ± 5.8	11.4 ± 3.5	3.8 ± 2.4	1.1 ± 1.8
5	Nimesulide 200 ppm	5.4 ± 3.3	25.8 ± 6.8	18.3 ± 6.2	5.0 ± 4.6	0.9 ± 1.0
6	Nimesulide 400 ppm	7.4 ± 2.9	35.9 ± 18.9	22.3 ± 10.6	3.6 ± 2.1	0.5 ± 0.8
7	Mofezolac 300 ppm + nimesulide 200 ppm	6.8 ± 4.7	37.3 ± 15.8[4]	18.5 ± 4.5	2.6 ± 1.1[4]	0.5 ± 0.5
8	Mofezolac 600 ppm + nimesulide 400 ppm	6.1 ± 2.9	29.8 ± 2.8[4]	17.0 ± 9.7	2.0 ± 1.6[4]	0.1 ± 0.4[4]
9	Indomethacin 10 ppm	4.9 ± 2.5	31.4 ± 9.1[4]	12.4 ± 6.8	2.0 ± 2.8[4]	0.1 ± 0.4[4]

[1]Mice were fed the basal diet or diets containing compounds starting at 7 weeks of age for 4 weeks. Animals in group 1 were sacrificed at the commencement.–[2]Data are mean ± SD values. The numbers of animals per group were 7 for group 1 and 8 for groups 2 to 9.–[3]$p < 0.01$ *vs.* the basal diet group (group 2).–[4]$p < 0.05$ *vs.* the basal diet group (group 2).

TABLE III – PROLIFERATION RATES OF POLYPS AND CRYPT EPITHELIUM IN THE SMALL INTESTINE AFTER 4 WEEKS OF TREATMENT WITH MOFEZOLAC AND/OR NIMESULIDE OR INDOMETHACIN IN *APC1309* MICE AND WILD-TYPE MICE

Group number	Treatment[1]	Genotype	BrdU labeling index (%)[2]	
			Polyps	Crypt epithelium
2	Basal diet	*Apc* heterozygous	31.7 ± 3.5	43.6 ± 6.4
3	Mofezolac 300 ppm	*Apc* heterozygous	26.2 ± 1.3[3]	36.0 ± 3.7
4	Mofezolac 600 ppm	*Apc* heterozygous	25.7 ± 4.5[3]	32.7 ± 2.6[3]
5	Nimesulide 200 ppm	*Apc* heterozygous	26.0 ± 2.0[3]	30.5 ± 2.5[4]
6	Nimesulide 400 ppm	*Apc* heterozygous	25.0 ± 3.3[3]	31.1 ± 3.0[4]
7	Mofezolac 300 ppm + nimesulide 200 ppm	*Apc* heterozygous	24.0 ± 1.2[4]	30.6 ± 2.3[4]
8	Mofezolac 600 ppm + nimesulide 400 ppm	*Apc* heterozygous	21.5 ± 2.0[4]	26.0 ± 3.7[4]
9	Indomethacin 10 ppm	*Apc* heterozygous	21.9 ± 2.3[4]	28.5 ± 3.9[4]
10	Basal diet	Wild-type		32.0 ± 1.4[3]
11	Mofezolac 600 ppm	Wild-type		31.7 ± 2.2[3]
12	Nimesulide 400 ppm	Wild-type		30.1 ± 2.0[4]
13	Mofezolac 600 ppm + nimesulide 400 ppm	Wild-type		29.2 ± 3.3[4]
14	Indomethacin 10 ppm	Wild-type		32.0 ± 2.3[3]

No statistical differences between the basal diet group (group 10) and any of the treated groups (groups 11 to 14) of wild-type mice.–[1]Mice were fed the basal diet or diets containing compounds starting at 7 weeks of age for 4 weeks. Animals in group 1 were sacrificed at the commencement.–[2]Data are mean ± SD values. The number of animals examined per group was 5.–[3]$p < 0.05$ *vs.* the basal diet group (group 2).–[4]$p < 0.01$ *vs.* the basal diet group (group 2).

might simply be due to the increased total dosage of inhibitors, this appears unlikely because 200 ppm nimesulide plus 300 ppm mofezolac was significantly effective at reducing mean polyp area even though 600 ppm mofezolac alone exerted only a nonsignificant suppressive effect. Our observations thus indicated that both COX-1 and COX-2 contribute to intestinal carcinogenesis.

A human colon carcinoma cell line transfected with cDNAs for either COX-1 or COX-2 expresses either isoform with increased growth rates in both cases, the stimulation being suppressed by indomethacin treatment.[25] An involvement of COX-1, comparable with that of the COX-2 isoform, in intestinal tumorigenesis in Min mice was also demonstrated on the basis of a genetic approach by Chulada *et al.*[14] We confirmed this using a pharmacologic approach with mofezolac against AOM-induced colonic ACFs in rats and intestinal polyps in *APC1309* mice.[15] PGE_2 levels in polyps are increased compared with those of the normal tissue and suppressed by COX-1 or COX-2 deficiency.[14] As with the Min mice, COX-1 is found in both normal tissue and polyps, while COX-2 is limited to polyps in *APC1309* mice (data not shown). In the present study, the crypt epithelial cells of *APC1309* mice treated with COX inhibitors and wild-type mice showed lower BrdU labeling indexes compared with those cells of *APC1309* mice fed with basal diet. From these findings, it is suggested that deficiency of the *Apc* gene stimulates cell proliferation of crypt epithelium in mice and that COX inhibitors suppress the increase in cell proliferation rate. This would be expected to reduce polyp growth and the reduction was suggested by a decrease in cell proliferation rate of the polyps in the present study. Recently, COX-1 and COX-2 inhibitors were reported to suppress tumor growth and metastasis in mice with established metastatic mammary tumors,[26] and genetic deficiency for either form reduced skin tumorigenesis in a mouse skin model.[27] Thus, both COXs may contribute to tumorigenesis in various organs.

The COX isoforms possibly compensate for lack of expression of the other.[28] Dual-COX inhibition would be expected to overcome such compensation in intestinal tumorigenesis and this may be one of the reasons for the combination effect. Recently, NSAID-induced gastric injury was found to require suppression of both COX-1 and COX-2 in rodents.[29] Actually, administration of the COX-1 selective inhibitor, mofezolac, did not induce any gastrointestinal side effects in the rats or mice.[15] In the present study, no lesions of the glandular stomach or small intestines were observed in any of the groups, including those receiving combined COX-1 and COX-2 inhibitors at doses sufficient to suppress intestinal polyp development. Obviously, it is of great interest to know whether the safety margin with combinations of COX-1 and COX-2 selective inhibitors might be wider than with dual-COX inhibitors. In addition, it remains unclear whether COX-1 and COX-2 isoforms exert their effects at different stages of intestinal carcinogenesis through different mechanisms. It is possible that the COX isoforms are involved in production of prostaglandins and contribute to carcinogenesis through different receptor-mediated pathways, for example, by impacting on EP_1, EP_2 and EP_4 receptors.[18–20]

To establish more effective and safer chemopreventive measures for colon carcinogenesis, further investigation of the effects of combinations of COX-1 and COX-2 selective inhibitors on colon cancer development is clearly warranted.

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Review

Mutagens formed from β-carbolines with aromatic amines

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Abstract

Norharman, widely distributed in our environment such as cigarette smoke and cooked foods, is not mutagenic to *Salmonella* strains, but becomes mutagenic to *Salmonella typhimurium* TA98 and YG1024 with S9 mix in the presence of aromatic amines, including aniline and *o*-toluidine. Therefore, we have designated norharman as a "co-mutagen". Since, humans are simultaneously exposed to norharman and aromatic amines in daily life, it is important to clarify the mechanisms of its co-mutagenic action to further understanding of the potential genotoxic effects in humans. Regarding the mechanisms of this action of norharman with aniline, a mutagenic compound, 9-(4′-aminophenyl)-9*H*-pyrido[3,4-*b*]indole[aminophenylnorharman (APNH)] is produced by their interaction, and converted to the hydroxyamino derivative which eventually forms the DNA adduct, dG-C8-APNH through possible ultimate reactive forms with esterification, and this induces mutations. Also other aminophenyl-β-carboline compounds, such as 9-(4′-amino-3′-methylphenyl)-9*H*-pyrido[3,4-*b*]indole[amino-3′-methylphenylnorharman (3′-AMPNH)], 9-(4′-amino-2′-methylphenyl)-9*H*-pyrido[3,4-*b*]indole [amino-2′-methylphenylnorharman (2′-AMPNH)], 9-(4′-aminophenyl)-1-methyl-9*H*-pyrido[3,4-*b*]indole[aminophenylharman (APH)] and 9-(4′-amino-3′-methylphenyl)-1-methyl-9*H*-pyrido[3,4-*b*]indole[amino-3′-methylphenylharman (AMPH)], have been found on reaction of norharman or harman with aniline or toluidine isomers. These compounds showed mutagenic and clastogenic actions in bacterial and mammalian cells. Among them, APNH demonstrated the most potent activity, and it was most extensively studied. When APNH was administered as a single dose to F344 rats, severe testicular toxicity was observed after 6 days. Moreover, liver preneoplastic lesions (GST-P-positive foci) in the liver clearly developed in animals fed 10–50 ppm of APNH in the diet for 4 weeks. Since, APNH was detected in 24 h urine of rats upon simultaneous administration with norharman and aniline by gavage, it is likely to be also produced from norharman and aniline in the human body. From these findings, it is suggested that aminophenyl-β-carboline derivatives may be classified as one of the novel types of endogenous mutagens and carcinogens.
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Keywords: Reviews; Mutagens; β-Carbolines; Norharman; Aniline

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Abbreviations: HAs, heterocyclic amines; Trp-P-1, 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole; Trp-P-2, 3-amino-1-methyl-5*H*-pyrido [4,3-*b*]indole; Norharman, 9*H*-pyrido[3,4-*b*]indole; Harman, 1-methyl-9*H*-pyrido[3,4-*b*]indole; APNH, 9-(4′-aminophenyl)-9*H*-pyrido[3,4-*b*]indole; 3′-AMPNH, 9-(4′-amino-3′-methylphenyl)-9*H*-pyrido[3,4-*b*]indole; 2′-AMPNH, 9-(4′-amino-2′-methylphenyl)-9*H*-pyrido[3,4-*b*]indole; APH, 9-(4′-aminophenyl)-1-methyl-9*H*-pyrido[3,4-*b*]indole; AMPH, 9-(4′-amino-3′-methylphenyl)-1-methyl-9*H*-pyrido[3,4-*b*]indole; MeIQx, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline; SCE, sister chromatid exchange; CHL, Chinese hamster lung; N-OH-APNH, hydroxyaminophenylnorharman; PHA, phenylhydroxylamine; dG-C8-APNH, $N^{4'}$-(2′-deoxyguanosin-8-yl)-9-(4′-aminophenyl)-9*H*-pyrido[3,4-*b*]indole; GST-P, glutathione-*S*-transferase placental form

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1. Introduction

More than 20 years ago, Nagao co-workers reported that the β-carboline compound, norharman (9*H*-pyrido[3,4-*b*]-indole), itself not mutagenic to *Salmonella* strains with or without S9 mix, becomes mutagenic to *Salmonella typhimurium* TA98 and YG1024 with S9 mix, when mixed with aromatic amines, such as aniline and toluidine isomers [1–3]. On the bases of these observations, we designated norharman is a "co-mutagen". Harman (1-methyl-9*H*-pyrido[3,4-*b*]indole), another β-carboline compound, has similarly co-mutagenic activity with aniline or *o*-toluidine [3].

Norharman and harman are produced by pyrolysis of L-tryptophan and have been reported to be present at much higher levels than mutagenic and carcinogenic HAs in cigarette smoke condensate and cooked foodstuffs [4]. The compounds were also detected in human urine samples [5,6]. Since, these β-carboline compounds were detected in both of urine samples collected from healthy volunteers eating normal diet and patients receiving parenteral alimentation, it is suggested that norharman and harman are probably produced endogenously in human body. Aniline and toluidine isomers are also present in cigarette smoke condensate and certain vegetables. These aromatic amines are ubiquitously utilized in various ways as industrial raw materials [7–9] and aniline is detectable in human urine and breast milk samples [10–12]. Therefore, it is likely that humans are simultaneously exposed to β-carbolines and aromatic amines in daily life and the clarification of the mechanisms of its co-mutagenic action is important in order to understand potential genotoxic effects in humans.

2. Structures of mutagens produced by norharman with aromatic amines

Since norharman showed mutagenic activity when mixed with aromatic amines and S9 mix, efforts were made to identify the mutagenic compound formed by reaction between norharman and aniline in the presence of S9 mix. After incubation, the reaction mixture was separated by HPLC on a semi-preparative ODS column with a gradient solvent system. Examination of mutagenicity in *S. typhimurium* YG1024 with S9 mix, revealed activity mainly in the fractions with retention times of 48–60 min (compound I). The mutagenic compound I was further isolated and purified by HPLC, and various spectral determinations performed including UV, mass and ^{1}H-NMR spectra. The UV absorption spectrum of the mutagenic compound I showed absorption maxima at 238, 287 and 356 nm, and its mass spectrum exhibited a molecular ion peak at *m/z* 259. With ^{1}H-NMR spectral data measured in acetone-d_6 indicated 7 of the 11 aromatic protons could be assigned to the norharman moiety and 4 protons to the aniline moiety. Based on spectral data, the mutagenic compound I was deduced to be a coupled compound of norharman and aniline, namely 9-(4′-aminophneyl)-9*H*-pyrido[3,4-*b*]indole [aminophenylnorharman (APNH)]. This assumption was confirmed by its chemical synthesis. The yield of APNH from norharman in the enzymatic reaction was around 0.01%. On the other hand, when the mutation assay of the each fractions collected from HPLC separation was carried out in absence of S9 mix, mutagenicity was detected in fractions with retention times of 60–68 min (compound II). Aromatic amine compounds need to be converted to an *N*-hydroxyamino derivative to cause mutagenicity [13] and compound II was tentatively considered to be hydroxyaminophenylnorharman (N-OH-APNH). To confirm its structure, N-OH-APNH was synthesized, and finally the structure of compound II, showing mutagenic activity without S9 mix, was concluded to be N-OH-APNH [14,15].

As mentioned above, the other aromatic amines, such as *o*- and *m*- but not *p*-toluidine, have also been shown to react with norharman to elicit mutagenicity in *S. typhimurium* TA98 and YG1024 in presence of S9 mix. Mutagenic compounds produced from norharman with *o*- or *m*-toluidine were isolated and their structures were determined. The mutagenic compounds produced from norharman with *o*-toluidine and *m*-toluidine were 9-(4′-amino-3′-methylphneyl)-9*H*-pyrido[3,4-*b*]indole [amino-3′-methylphenylnorharman (3′-AMPNH)] and 9-(4′-amino-2′-methylphenyl)-9*H*-pyrido[3,4-*b*]indole [amino-2′-methylphenylnorharman (2′-AMPNH)], respectively [16]. The yields of these compounds in the incubation mix were around 0.06 and 0.02%, respectively.

It already demonstrated that also harman, another β-carboline compound, causes co-mutagenicity in *S. typhimurium* TA98 in presence of S9 mix and aniline [3]. Therefore, it is likely that coupled mutagenic compounds of harman with aniline or *o*-toluidine are produced in the reaction mixtures. Two mutagenic compounds were identified so far, namely 9-(4′-aminophenyl)-1-methyl-9*H*-pyrido[3,4-*b*]-indole [aminophenylharman (APH)] formed in the reaction of harman with aniline, and 9-(4′-amino-3′-methylphenyl)-1-methyl-9*H*-pyrido[3,4-*b*]indole [amino-3′-methylphenylharman (AMPH)] from the reaction with *o*-toluidine. The yields of these compounds were around 0.0025% for APH and 0.005% for AMPH. The chemical structures of the five aminophenyl-β-carboline derivatives, APNH, 3′-AMPNH, 2′-AMPNH, APH and AMPH are given in Fig. 1.

3. Genotoxic activities of aminophenyl-β-carboline derivatives

Chemically synthesized APNH, 3′-AMPNH, 2′-AMPNH, APH and AMPH caused higher mutagenicity in *S. typhimurium* TA98 and YG1024 (which detect frameshift mutations) than in the strains TA100 and YG1029 (which are sensitive to base pair mutations) in presence of S9 mix.

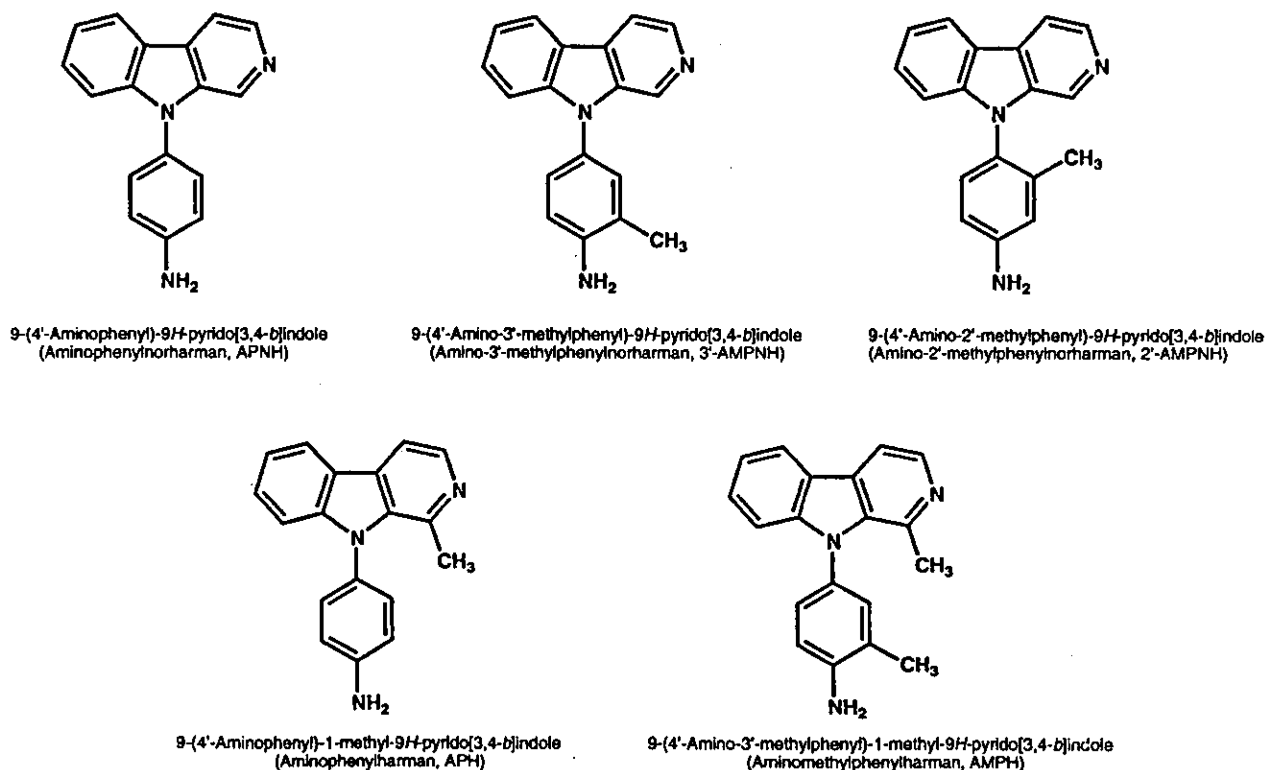

Fig. 1. Chemical structures of aminophenyl-β-carboline derivatives.

Moreover, YG1024, a TA98 derivative with high acetyltransferase activity proved more sensitive than the parent strain. This observation suggest that acetyltransferase is required for the mutagenicity of these aminophenyl-β-carboline derivatives. The mutagenic activity of APNH, 2′-AMPNH, 3′-AMPNH and three HAs are shown in Table 1 [14–17]. Among the aminophenyl-β-carboline derivatives studied, APNH showed the strongest mutagenicity with a value comparable to those for 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), 3-amino-1-methyl-5*H*-pyrido [4, 3-*b*]indole (Trp-P-2) and about 500 times higher than that of benzo[*a*]pyrene [17]. The mutagenic activities of other derivatives including APH and AMPH, were 100–200 times lower than those of APNH. In absence of S9 mix, aminophenyl-β-carboline derivatives were not mutagenic in either strain.

Table 1
Mutagenicities of aminophenyl-β-carboline derivatives and HAs in *Salmonella* strains in presence of S9 mix [14–17]

	Mutagenicity (revertants/μg)		
	TA98	TA100	YG1024
APNH[a]	187000	1230	1783000
2′-AMPNH[a]	140	16	3000
3′-AMPNH[a]	41000	2	698000
MeIQx	145000	14000	3500000
Trp-P-2	104200	1800	77000
Glu-P-1	49000	3200	2500000

[a] S9 concentration was used for 5 μl per plate.

In subsequent experiments the DNA adduct formation by norharman in presence of aniline or toluidine isomers (*o*-, *m*- and *p*-toluidines) was analyzed. After incubation of *S. typhimurium* TA98 with norharman plus aniline, or each of the three kinds of toluidine isomers in the presence of S9 mix, DNA adducts were analyzed by the ^{32}P-postlabeling method under modified adduct intensification conditions. Formation of DNA adducts was clearly observed with mixtures of norharman plus aniline or *o*- or *m*-toluidine but not in the case of *p*-toluidine. Three adduct spots (two major and one minor) were found in the reaction mixture of norharman plus aniline, furthermore two major and one minor spots for norharman plus *o*-toluidine, and a single spot for norharman plus *m*-toluidine were detected [18]. In contrast, no adduct spots were produced by incubation of any of the aromatic amines or by norharman alone. The mutagenicities of norharman (100 μg per plate) in presence of aromatic amines (200 μg per plate), such as aniline or toluidine isomers, towards *S. typhimurium* TA98 with S9 mix, as well as the DNA adduct levels are shown in Table 2. Mixtures of norharman with aniline and *o*- or *m*-toluidine, caused mutagenicity, whereas no mutagenic activity was observed with the combination of norharman and *p*-toluidine, or with any of the compounds alone. Thus, the DNA adduct formation by norharman with aromatic amines correlated well with the co-mutagenic action in

Table 2
Relationship between the co-mutagenic action and DNA adduct formation in *S. typhimurium* TA98 seen after incubation of norharman with aromatic amines and S9 mix [18]

	Mutagenicities[a] (revertants per plate)	Adduct levels[b] (adducts/10^8 nucleotides)
Norharman + aniline	6610 ± 1210	10.8 ± 2.27
Norharman + *o*-toluidine	6990 ± 1043	3.74 ± 1.71
Norharman + *m*-toluidine	62 ± 6	0.04 ± 0.01
Norharman + *p*-toluidine	0	N.D.[c]
Norharman	0	N.D.[c]
Aniline	0	N.D.[c]
o-Toluidine	0	N.D.[c]
m-Toluidine	0	N.D.[c]
p-Toluidine	0	N.D.[c]

Numbers of revertants and RALs in *S. typhimurium* TA98 are mean±S.D. values.

[a] Mutagenicity of norharman in combination with aromatic amines examined in *S. typhimurium* TA98 with S9 mix. The doses of norharman and aromatic amines were 200 and 100 μg per plate, respectively.

[b] DNA adduct levels were analyzed by ^{32}P-postlabeling method under modified adduct intensification conditions.

[c] N.D.: not detectable levels (<$1/10^{10}$ nucleotides) of aminophenyl-β-carboline–DNA adducts.

S. typhimurium TA98 [18]. Moreover, the chemical structure of the major APNH–DNA adduct was concluded to be $N^{4'}$-(2′-deoxyguanosin-8-yl)-9-(4′-aminophenyl)-9*H*-pyrido-[3,4-*b*]indole (dG-C8-APNH) using the ^{32}P-postlabeling method and various spectrometry techniques [19]. APNH–DNA adduct formation was observed in various organs of F344 rats fed 40 ppm of APNH for 4 weeks, the highest levels were detected in the liver and colon (1.31 ± 0.26 and 1.32 ± 0.11 adducts/10^7 nucleotides, respectively) [19].

Recently, Ohe et al. reported that APNH, 3′-AMPNH and APH induced sister chromatid exchange (SCE) in Chinese hamster lung (CHL) cells [20]. The cells were incubated with the individual compounds in presence of S9 mix, and SCEs were induced in a dose-dependent manner at concentrations between 0.00125 and 0.01 μg/ml for APNH, and between 0.3125 and 5 μg/ml for 3′-AMPNH and APH. The approximate doses leading to three-fold increase over the control level were 0.05 for APNH, 0.51 for 3′-AMPNH and 1.7 μg/ml for APH. In addition, APNH induced chromosome aberrations in CHL cells at concentrations between 0.00125 and 0.04 μg/ml, the potency of SCE induction and clastogenic activity was much stronger than that seen with potent model clastogens, such as actinomycin D, mitomycin C or 1,8-dinitropyrene [20].

4. The mechanisms of APNH formation

In a number of experiments, the enzymes in rat liver involved in the in vitro formation of APNH from norharman and aniline were investigated. The microsomal fraction was clearly active, whereas no APNH formation was observed with cytosol fraction. Addition of a P450 inhibitor (SKF-525A) to the reaction mixture of norharman, aniline and the microsomal fraction resulted in a decrease to approximately 40% of the APNH level in the reaction mixture without the inhibitor. These findings indicated that P450s mediated the formation of APNH from norharman and aniline [21]. In addition, APNH was also detected in presence of the microsomal fraction derived from human liver. The mechanisms of the reaction are not fully known yet, but, Guengerich suggested the possibility that the two amines are brought together in a P450 complex so an *ipso* attack on the aniline can occur [22]. On the other hand, it has been reported that a mixture of norharman and phenylhydroxylamine (PHA) which is a *N*-hydroxy-derivative of aniline, in the presence of S9 mix, causes three times higher mutagenicity than the mixture of norharman and aniline [3]. Moreover, we confirmed that APNH was formed in the reaction mixture of norharman and PHA with S9 mix. Thus, an other possible mechanism of formation of APNH can be postulated: first, aniline is oxidized to form PHA by P450(s), which might be converted to a phenyl nitrenium cation by further enzymatic reactions. A nitrenium carbo-cation at the C-4 position derived from nitreniumion, might be produced, and could bind to norharman to form APNH (unpublished data). It remains to be clarified which P450 enzyme(s) is (are) involved in the formation of APNH. On the other hand, it is also possible that other types of enzymes are involved in the reaction of norharman and aniline.

The in vivo formation of APNH was studied by detection of APNH in the urine samples using gas chromatography with nitrogen-phosphorous selective detector, a selective and sensitive method for the detection of HAs. A mixture of norharman and aniline was administered by gavage to F344 rats pretreated with phenobarbital and β-naphthoflavone, then the amount of APNH in 24 h urine was analyzed. In urine samples from rats administered 45 mg/kg or 90 mg/kg each of norharman and aniline, 11.5 ± 8.6 ng was detected in the 24 h urine and 19.6 ± 16.9 ng in the 24 h urine [21]. In contrast, APNH was not detected in urine samples from rats exposed to norharman or aniline, alone. Moreover, the amount of APNH was about 2–3-fold increased in urine samples treated with 1N hydrochloric acid at 60 °C for 5 h for hydrolysis of APNH conjugates. As mentioned above, both, norharman and aniline are present in our environment, therefore, it is likely that APNH is similarly produced in humans.

5. Metabolism of APNH

Since APNH shows higher mutagenic activity in *S. typhimurium* YG1024 with high acetyltransferase than in TA98 in the presence of S9 mix, the compound may be metabolized by cytochrome P-450 enzymes with conversion of an exocyclic amino group to a hydroxyamino group, then further activated to form the *N*-acetoxy derivative by the action of acetyltransferase, as is the case for HAs [13,17].

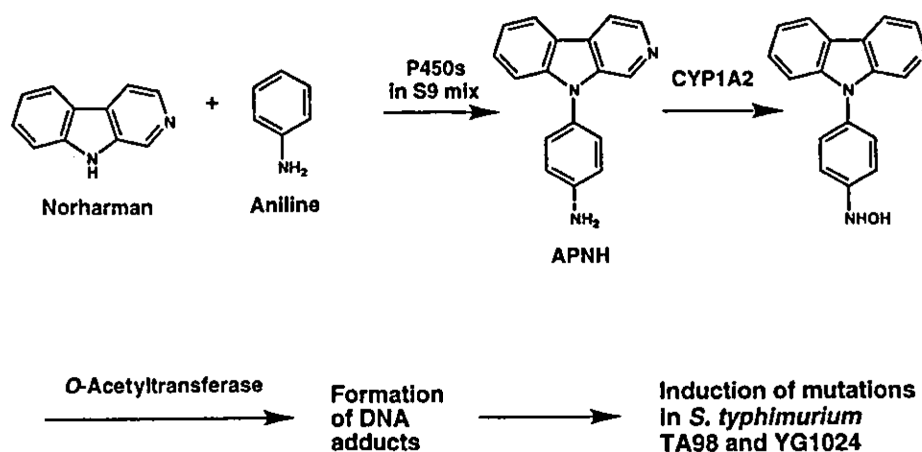

Fig. 2. Schematic illustration of the reaction of norharman with aniline in the presence of S9 mix leading to appearance of mutagenicity.

For most HAs, except for Trp-P-1, the principal cytochrome P-450 isozyme responsible for *N*-hydroxylation is known to be CYP1A2 [23]. Using genetically engineered *S. typhimurium* OY 1002 which contains one of the various forms of human cytochrome P-450 and NADPH–P-450 reductase, APNH was found to be mainly *N*-hydroxylated by CYP1A2, also CYP2C9 elicited weak activity, but other isozymes, including CYP1A1, 1B1, 2D6, 2E1 and 3A4, showed no ability to *N*-hydroxylate APNH [24]. In addition, APNH yielded identical DNA adducts in *S. typhimurium* YG1024 as those observed with N-OH-APNH and also the mixture of norharman and aniline [14]. Moreover, it was reported that 3′-AMPNH produces the same DNA adducts in *S. typhimurium* YG1024 as those observed with the mixture of norharman and *o*-toluidine [16]. From these observations, possible mechanisms of the co-mutagenic action of norharman with aniline are suggested, as follows (Fig. 2): (i) the coupled mutagenic compound, APNH, is formed by an enzymatic reaction, presumably with P450(s); (ii) subsequently, the exocyclic amino group is metabolically activated by CYP1A2 and the *N*-hydroxyamino derivative further metabolized to the *N*-acetoxy form by the action of acetyltransferase. The ultimate metabolite produces DNA adducts which induce mutations in *Salmonella* strains. This might also be the case for compounds generated by norharman or harman with other aromatic amines.

6. In vivo toxicity of APNH in F344 rats

Since humans may be continuously exposed to both norharman and aniline in their daily life, the possible toxic and carcinogenic effects of APNH in mammals should be studied. To assess this question, a short-term experiment was conducted in F344 rats. Ten-week-old males were treated with a single intragastric injection of APNH at doses of 45 mg/kg or 90 mg/kg body weight and sacrificed 1, 3 or 6 days thereafter. With the highest dose, the food intake was suppressed and body and testis weights were decreased by day 6. Histopathological examinations clearly demonstrated that APNH induced severe testicular damage, such as vacuolation of Sertoli cells, appearance of multinucleated giant cells and loss of round spermatids and spermatogonia in the seminiferous tubules (Fig. 3) [25]. However, Leydig cells

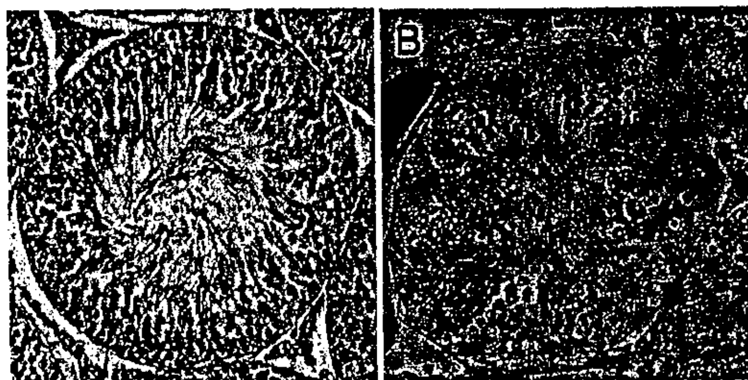

Fig. 3. Histopathological appearance of seminiferous tubules in rats 6 days after administration of water (A) or 90 mg/kg of APNH (B) (H&E, original magnification 20×).