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Review

Chemical and Biological Approaches for Detecting Environmental Causes of Cancer

Keiji Wakabayashi[1]

National Cancer Center Research Institute, Tokyo, Japan

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Environmental factors exert important influences on cancer development in humans. Cancer is now one of the major causes of death in our country, and it is necessary to search for mutagens and carcinogens in our environment to obtain information for evaluation of relationships with cancer patterns. My group has been involved in the identification of novel types of environmental mutagens and carcinogens and also in the study of mechanisms underlying colon carcinogenesis in rodents. The following results were obtained: 1) various kinds of mutagenic and carcinogenic heterocyclic amines were identified in cooked foods; 2) aminophenylnorharman was identified as a mutagenic compound formed from norharman and aniline in the presence of S9 mix; 3) mutagens and carcinogens were shown to be produced by the reaction of environmental aromatic compounds with nitrite; 4) eight kinds of phenylbenzotriazole compounds were isolated and identified as mutagens from river water in Japan; 5) enhancing effects of hyperlipidemia were demonstrated in intestinal carcinogenesis. In this review, roles of environmental factors in cancer development and related approaches to possible cancer prevention in humans are discussed.

Key words: heterocyclic amines, aminophenylnorharman, nitrosatable mutagen/carcinogen precursors, phenylbenzotriazole compounds, colon carcinogenesis

Introduction

It is now abundantly evident that human cancers have multiple genetic and epigenetic alterations in genes that affect cell proliferation, apoptosis resistance and metastasis. Cancers with more malignant properties are generally associated with larger numbers of such alterations.

In addition to cigarette smoking, diet plays an important role in cancer development (1). Excess intake of total calories and fat increase the likelihood of colon and mammary cancer, and that of sodium chloride promotes gastric cancer development (2–4). Food also contains mutagenic and carcinogenic compounds as very minor components (5,6). These mutagenic and carcinogenic compounds exist in several situations: 1) as contaminants due to mould growth, 2) as edible plant components, 3) as substances formed during storage and fermentation of food, some of which are nitrosatable substances, 4) as products of cooking, and 5) as food additives, mainly for preservation. Moreover, food also contains many substances which may suppress formation of carcinogenic compounds and cancer development.

The multiple genetic and epigenetic alterations in carcinomas are presumably caused by different environmental carcinogens, as well as endogenous carcinogenic substances, most of which exist only at low levels and cannot play major roles alone. As environmental carcinogens, polycyclic aromatic hydrocarbons, *N*-nitroso compounds, mycotoxins and heterocyclic amines (HCAs) are typical examples, existing in our environment as contaminants in food, air and water. Endogenous genotoxic agents, including oxygen radicals, can also modify DNA bases, as represented by the formation of 8-hydroxyguanine and thymine glycol (7,8). Under the acidic conditions in the stomach, nitrite reacts with secondary amines to produce carcinogenic nitrosamines (9,10). In addition, nitric oxide synthase produces NO from L-arginine, and this NO may also be involved in endogenous formation of nitroso compounds (11).

My group has been involved in the identification of environmental mutagens and carcinogens and also endogenous mutagens and carcinogens. Moreover, detailed studies of how hyperlipidemia, one of the major characteristics of the metabolic syndrome, is linked to increased risk of colon cancer have been performed. In this review, chemical and biological properties and possible formation pathways of identified mutagens and carcinogens are reported and mechanisms underlying enhancing effects of hyperlipidemic on colon carcinogenesis are also described. Moreover, contributions of environmental and endogenous factors to human car-

[1]Correspondence to: Keiji Wakabayashi, Office of the Director, National Cancer Center Research Institute, 1-1 Tsukiji 5-chome, Chuo-ku, Tokyo 104-0045, Japan. Tel: +81-3-3542-2511, Fax: +81-3-3248-0326, E-mail: kwakabay@ncc.go.jp

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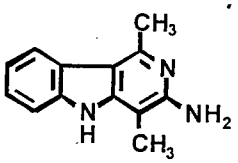
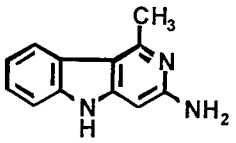
cinogenesis and possible prevention methods are discussed.

Identification of Mutagenic and Carcinogenic Heterocyclic Amines in Cooked Foods

In the 1970–80s, a series of mutagenic substances, namely HCAs, were discovered in foodstuffs by scientists in Japan and the United States (6,12–15). More than 20 mutagenic HCAs are now known to be formed in meat and fish under common cooking conditions. Among these HCAs, ten HCAs have been demonstrated to induce cancer in the liver, colon, prostate, mammary gland, for example, in experimental animals including rats and mice. In these cancers, *Apc*, *β-catenin*, *p53* and K-, H-*ras* gene mutations were detected. The structures of the ten carcinogenic HCAs are shown in Fig. 1. These compounds are formed by pyrolysis of amino acids (16,17) or creatin(in)e, free amino acids and sugars (18). The most abundant HCAs in cooked foods are PhIP and MeIQx. Based on the available data, human exposure has been estimated to be between 0.1 and 13.8 μg for PhIP, and 0.2 and 2.6 μg for MeIQx per day (19). To elucidate exposure levels of HCAs in humans, direct measurements have been performed using various human biological samples, such as urine, breast milk and hair. Some HCAs, including MeIQx, PhIP, Trp-P-1



3-Amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-1)



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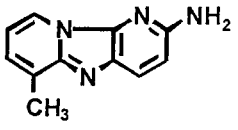
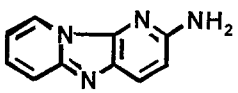


2-Amino-6-methyldipyrido[1,2-*a*:3',2'-*d*]imidazole (Glu-P-1)



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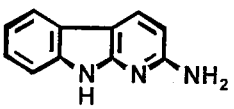

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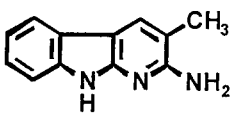

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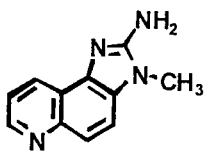
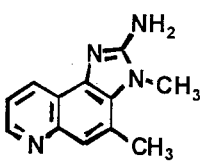


2-Amino-3-methylimidazo[4,5-*f*]quinoline (IQ)



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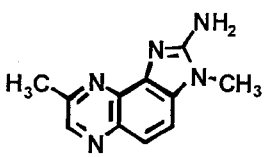
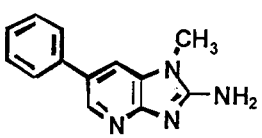


2-Amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx)



2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP)

Fig. 1. Structures of mutagenic and carcinogenic HCAs.

and Trp-P-2, have been detected in urine samples from volunteers consuming a normal diet (20) and PhIP has been found in the milk and hair of healthy women (21,22). In contrast, no HCAs were detected in patients receiving parental alimentation (20).

Several HCA-DNA adducts have already been characterized in experimental animals, the major forms being due to binding of the exocyclic amino groups of HCAs with C8 atoms of guanine (dG-C8-HCA). dG-C8-MeIQx has been detected by ^{32}P-postlabeling in DNA samples extracted from human colon, rectum and kidney specimens derived from surgical and autopsy samples (23). Similarly, another report documented dG-C8-PhIP in human colon samples (24). Several epidemiological studies have provided evidence of positive associations between higher consumption of well-done red meat and risk of colon (25–27), breast (28,29), and lung cancer (30). Moreover, early studies showed a significantly increased risk of colorectal cancer with consumption of well-done meat in individuals with a rapid phenotype for CYP1A2 or NAT2. Combination of CYP1A2 and NAT2 rapid phenotypes and well-done meat consumption resulted in a 6-fold increased risk in one investigation (31). The available reports thus suggest that high HCA exposure is associated with an elevated risk of colorectal cancer, especially in subgroups which are genetically susceptible with regard to metabolic and detoxification enzymatic activity.

Mutagenic and Carcinogenic Aminopheyl-β-carboline Compounds Formed from β-Carbolines and Aromatic Amines

β-Carbolines (norharman and harman) and aromatic amines (aniline and *o*-toluidine) are distributed widely in our environment, including cigarette smoke and cooked foods, so that humans are continuously exposed to these during daily life. In 1977, Dr. Nagao *et al.* reported that mutagenicity in *S.typhimurium* TA98 occurs when β-carbolines and aromatic amines co-exist in the presence of a metabolic activation system (S9 mix) (32). Subsequently, we found that a novel type of mutagenic HCA, aminophenylnorharman (APNH) [9-(4′-aminophenyl)-9*H*-pyrido[3,4-*b*]indole] is produced from norharman and aniline under these conditions (Fig. 2) (33). Similarly, other mutagenic amino-β-carboline compounds were produced from β-carboines and aromatic amines with S9 mix; aminomethylphenylnorharman [9-(4′-amino-3′-methylphenyl)-9*H*-pyrido[3,4-*b*]indole] from norharman and *o*-toluidine; aminophenylharman [9-(4′-aminophenyl)-1-methyl-9*H*-pyrido[3,4-*b*]indole] from harman and aniline; and aminomethylphenylharman [9-(4′-amino-3′-methylphenyl)-1-methyl-9*H*-pyrido[3,4-*b*]indole] from harman and *o*-toluidine (34). APNH is mutagenic not only in bacteria but also in mammalian cells and induces liver and colon cancers in rats (35). We identified CYP3A4 and CYP1A2 as the enzymes mediating APNH formation (36). Recently, this compound was also detected in 24-h urine samples from healthy volunteers (37) and therefore could play an important role in human carcinogenesis as a new type of endogenous mutagen/carcinogen.

Nitrosatable Mutagen/Carcinogen Precursors in Foodstuffs

Nitrate intake is associated with gastric cancer mortality in various countries (38,39). After ingestion, nitrate is excreted into saliva and is converted to nitrite by the nitrate reductase of bacteria in the oral cavity. Nitrite then reacts with secondary amines to produce N-nitroso compounds under acidic conditions. Direct-acting N-nitroso compounds such as *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine and *N*-methyl-*N*-nitrosourea are known to induce cancer in the glandular stomach of experimental animals (40,41). Thus, we have been engaged in searches for foodstuffs which show direct-acting mutagenicity upon nitrite treatment under acidic conditions.

In this way, soy sauce and vegetables were found to show direct-acting mutagenicity in *S.typhimurium* TA100 after nitrite treatment. Furthermore, several nitrosatable mutagen precursors could be isolated having structures of phenol and indole derivatives, such as tyramine, (−)-(1*S*,3*S*)-1-methyl-1,2,3,4-tetrahydro-β-

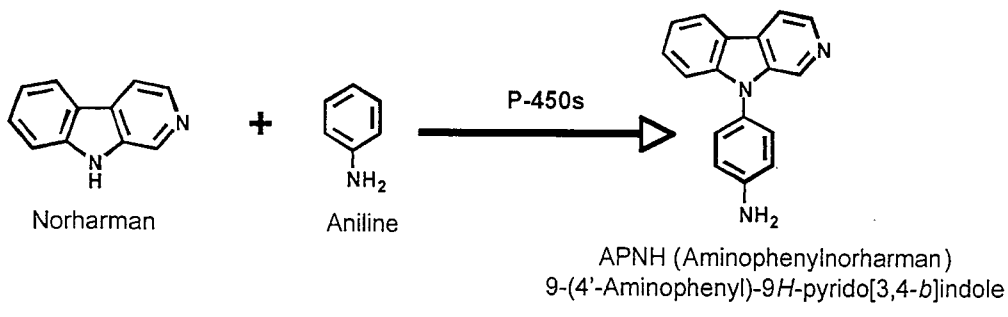


Fig. 2. Formation of APNH from norharman and aniline.

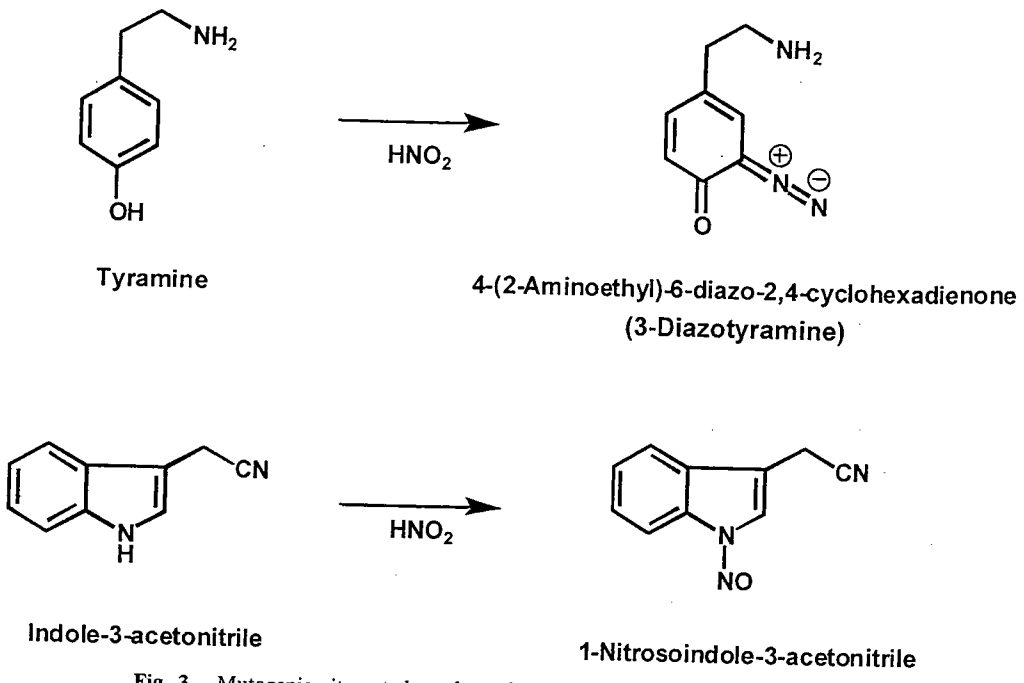


Fig. 3. Mutagenic nitrosated products from tyramine and indole-3-acetonitrile.

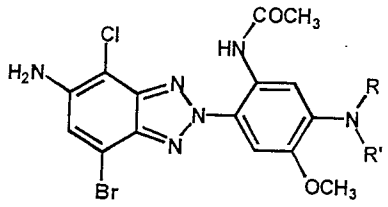
carboline-3-carboxylic acid, indole-3-acetonitrile, 4-methoxyindole-3-acetonitrile and 4-methoxyindole-3-aldehyde (42–44). One nitrosated product of tyramine was 3-diazotyramine, while indole-3-acetonitrile gave rise to N-1 nitrosated products (Fig. 3). 3-Diazotyramine and 1-nitrosoindole-3-acetonitrile could be demonstrated to form DNA adducts and induce mutations not only in bacteria strains, but also in cultured mammalian cells (44). Moreover, the diazo compound formed from tyramine with nitrite induced cancer in the oral cavity of rats (45). It is necessary to study the involvement of nitrosated products of phenolic compounds and those of indole compounds in gastric cancer development.

Identification of Mutagenic Phenylbenzotriazole Compounds in River Water

Surface waters are essential natural resources for drinking water and for industrial, agricultural, and recreational activities. The Yodo River system flows through Kyoto and Osaka prefectures in Japan, and water from this river system is used for water consumption for more than 13 million people living in the Osaka area. In 1982, Muraoka *et al.* (46) found the Katsura River, one of the principal components of the Yodo River system, to be polluted with mutagenic substances. Subsequently, many researchers have reported that water samples from the Yodo River system, including the Katsura and Nishitakase tributaries, contain mutagenic substances (47–50).

For elucidation of the chemical structures of major mutagens in the Yodo River system, blue cotton, to which the blue pigment, copper phthalocyaninetrisulfonate, is covalently bonded, was hung at a site below a sewage treatment plant in the Nishitakase River (51). The adsorbed materials were extracted with a mixture of methanol and ammonia, and separated by column chromatography using Sephadex LH-20 and HPLC. In consequence, three mutagenic compounds were isolated, which accounted for 21%, 17%, and 12% of the total mutagenic activities of river water samples, respectively. The chemical structures of these mutagens were determined to be 2-[2-(acetylamino)-4-[bis(2-methoxyethyl) amino]-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2*H*-benzotriazole (PBTA-1) (51), 2-[2-(acetylamino)-4-[*N*-(2-cyanoethyl) ethylamino]-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2*H*-benzotriazole (PBTA-2) (52), and 2-[2-(acetylamino)-4-amino-5-methoxyphenyl] -5-amino-7-bromo-4-chloro-2*H*-benzotriazole (PBTA-4) (53), on the basis of various spectral data and synthetic studies. Furthermore, several other PBTA compounds were identified as mutagens from river water samples, using a similar method (54). Chemical structures of mutagenic PBTA compounds are shown in Fig. 4.

On the basis of the synthetic studies, these PBTA congeners were thought to be formed from the corresponding azo compounds, which have a 2-[(2-bromo-4,6-dinitrophenyl)azo]-4-methoxyacetanilide moiety in their structures, via reduction with sodium hydrosulfite and subsequent chlorination with sodium hypochlorite (54). These azo compounds are very commonly used in dyeing factories around the world. Reducing agents



Compound		Revertants / μg*
PBTA-1	R:$C_2H_4OCH_3$ R':$C_2H_4OCH_3$	3,000,000
PBTA-2	R:C_2H_4CN R':C_2H_5	3,200,000
PBTA-3	R:C_2H_4OH R':H	3,000,000
PBTA-4	R:H R':H	7,800,000
PBTA-5	R:$C_2H_4OCOCH_3$ R':$C_2H_4OCOCH_3$	723,000
PBTA-6	R:C_2H_4OH R':C_2H_4OH	485,000
PBTA-7	R:C_2H_5 R':C_2H_5	2,980,000
PBTA-8	R:$CH_2CH=CH_2$ R':$CH_2CH=CH_2$	4,390,000

*S. typhimurium YG1024 with S9 mix

Fig. 4. Structures of mutagenic PBTAs isolated from river water and their mutagenicity.

such as sodium hydrosulfite are generally employed to help in discharging printing and bleaching materials in textile dyeing factories. Sodium hypochlorite is commonly used for bleaching in textile production and decolorization of wastewater and is also mainly utilized in sewage plants for disinfection purposes.

PBTA congeners were detected in the water concentrates from rivers flowing in Kyoto, Osaka, Aichi and Fukui prefectures, and the concentrates showed strong mutagenicity toward *S. typhimurium* YG1024 in the presence of S9 mix (54). All water concentrates were collected at sites below textile dyeing factories or municipal sewage plants treating domestic waste and effluents from textile dyeing factories. The highest level of a PBTA congener was detected in the Asuwa River in Fukui prefecture for PBTA-6 at 468 ng/g of blue rayon, accounting for 39% of the total mutagenic activity of the river water sample (54). High levels of PBTA-6 were also detected in many other river water samples, whereas PBTA-5 was lacking. In most textile dyeing factories, wastewater is treated with flocculants under alkaline conditions to remove dissolved and suspended substances prior to being discharged into surface water. Because PBTA-6 is easily formed from PBTA-5 by hydrolysis under alkaline conditions, PBTA-5 might be transformed to PBTA-6 in the wastewater treatment process in textile dyeing factories.

Mutagenic potencies of PBTA derivatives toward YG1024, an *O*-acetyltransferase-overproducing derivative of TA98, with S9 mix are shown in Fig. 4 (54). As a genotoxicity assay using mammalian cells, the *in vitro* micronucleus test was conducted on PBTA-1 and PBTA-2 with and without S9 mix in two Chinese hamster cell lines, CHL and V79-MZ (55, 56). PBTA-2 was a strong inducer of binucleated polynuclear and karyorrhectic cells in both cell lines, pointing to a potential to cause polyploidy. PBTA-1 showed clear positive results only in the absence of S9 mix and only in V79-MZ cells, inducing aneuploidy. In addition, genotoxicity of PBTA-6 was demonstrated in goldfish (*Carassius auratus*) by a micronucleus test and single-cell gel electrophoresis (comet assay) (57).

To assess risks of PBTA congeners to aquatic biota and human health, further studies on their biological features, including carcinogenicity and bioaccumulation, of these chemicals are necessary. In addition, quantitative studies of these compounds in river waters are required, and exposure levels of aquatic organisms and human to those compounds need to be clarified.

Enhancing Effects of Hyperlipidemia in Intestinal Carcinogenesis

The incidence and mortality of colon cancer are increasing in developed countries, including Japan. Intake of high fat diet, i.e., western-style diet, obesity and hyperlipidemia are linked to the rising risk of colorectal tumor (58–60) and one of our major interests is to clarify the mechanism whereby hyperlipidemia increases the likelihood of colorectal tumor development.

Recently, we reported an age-dependent hyperlipi-

demic state to exist in *adenomatous polyposis coli* (*Apc*)-deficient Min and Apc^{1309} mice, animal models of familial adenomatous polyposis (FAP) (61,62). In these *Apc*-deficient mice, serum TG levels were remarkably increased to ~10-fold the value observed in wild-type mice (Fig. 5) (61). In Min mice, high populations of very low-density lipoprotein (~50%) and low-density lipoprotein (~25%) were observed; in contrast to the predominance of high-density lipoproteins (~80%) in wild-type mice (61-63).

In addition to high concentrations of lipid in the serum, lipid accumulation in the liver, steatosis, was observed in Min mice, as confirmed by staining frozen sections with Oil-red O (61). Moreover, lipid accumulation in intestinal mucosa and polyps in Min mice was also evident on Oil-red O staining and electron microscopy (Fig. 6) and numbers of large lipid droplets were found in the epithelial cells in the upper parts of Min mice polyps (64). Small lipid droplets were also observed at the tips of the villi in non-tumorous parts of the small intestine of Min mice, but only very limited lipid deposition was seen in the villi of wild-type mice. Interestingly, overexpression of low-density lipoprotein receptor (LDLR) was observed in the same area where lipid droplets were observed (64). Real time-PCR analysis revealed that the LDLR mRNA levels in the intestinal polyps of Min mice were ~3 times higher than those in non-tumorous parts. It has been reported that the LDLR regulates most essential fatty acid uptake as well as cholesterol uptake into cells (65). We therefore speculated that a hyperlipidemic state and the lipid accumulation in the intestinal polyps may play an important role in intestinal polyp formation in *Apc*-deficient mice.

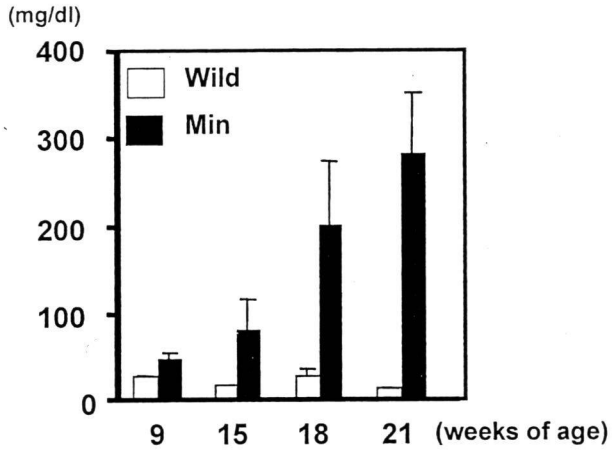


Fig. 5. Serum TG levels in Min and wild-type mice. Serum levels of TG were measured at the age of indicating weeks in Min mice (■) and wild-type (□) mice.

TG is hydrolyzed by lipoprotein lipase (LPL) to free fatty acids and monoacylglycerol. Physiologically, a decrease in or deficiency of LPL is associated with hyperlipidemic state (66) and *Apc*-deficient mice show decreased expression levels for LPL mRNA in the liver and small intestine. Thus, low LPL could be the reason for the hypertriglyceridemic state. No obvious differences were found for mRNA levels for fatty acid synthase, stearoyl-CoA desaturase-1, acyl-CoA oxidase, carnitine palmitoyl transferase-1 and phosphoenolpyruvate carboxykinase, enzymes involved in hydrolysis of TGs, lipogenesis, β-oxidation and glucose homeostasis in *Apc*-deficient mice.

In further experimentation, we induced LPL mRNA with a peroxisome proliferator-activated receptor (PPAR)-α agonist, bezafibrate, and a -γ agonist, pioglitazone, as well as a selective LPL-inducing agent, NO-1886, which lacks potential for the PPAR-α and -γ

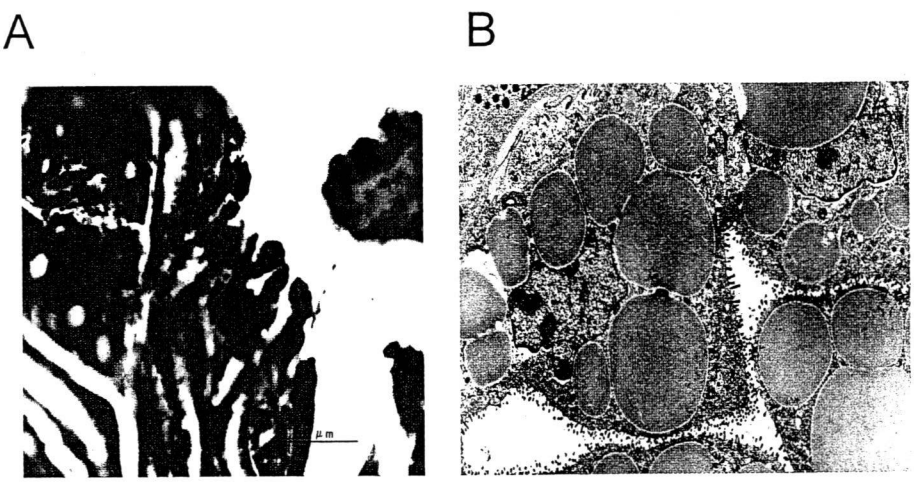


Fig. 6. Lipid accumulation in intestinal mucosa and polyps in Min mice. (A) Frozen sections of intestinal polyps were stained with Oil-red O, and red spots indicate lipids. (B) Transmission electron microscopy of epithelial cells of polyp part of the small intestine in Min mice revealed accumulation of lipid droplets. Magnification ×1,800.

activity, and demonstrated that all of these LPL inducers successfully suppressed the hyperlipidemic state, steatosis of the liver and intestinal polyp formation (61–63).

Plasminogen activator inhibitor-1 (Pai-1), an adipocytokine, is a direct binding primary inhibitor of plasminogen activators, uPA and tPA, and is induced by TG, VLDL (TG rich lipoprotein), TGFβ, various growth factors, tumor suppressor p53 and NFκB (67–69). Moreover, a VLDL response element exists in the PAI-1 promoter region that mediates VLDL-induced PAI-1 transcription in endothelial cells (70). Of note, PAI-1 is significantly overexpressed in neoplastic tissue in the human colon (71). Thus, we examined the levels of serum Pai-1 in 15-week-old male Min mice and found elevation to 8 times that in wild-type mice (72). Similarly, hepatic Pai-1 mRNA levels were increased 11-fold. Immunostaining revealed Pai-1 to be strongly expressed in small intestinal epithelial cells of Min mice. Administration of a PAI-1 inhibitor, SK-216, at 50 and 100 ppm doses in the diet for 9 weeks significantly reduced total numbers of intestinal polyps to 64 and 56 % of the untreated group value, respectively (72). Administration of SK-116, another PAI-1 inhibitor, similarly reduced the total numbers of intestinal polyps.

Based on these results, suppression of serum lipid levels by increasing LPL activity and inhibition of adipocytokines may contribute to reduction of intestinal polyp formation with *Apc*-deficiency, and the available information would also indicate that hyperlipidemia may contribute to development of human colon cancer.

Preventive Measures for Reducing Risk with Environmental and Endogenous Factors in Cancer Development

Humans are continuously exposed to various kinds of endogenous and exogenous mutagens/carcinogens. Even though the levels of each individual compound are very low, mutagens and carcinogens could interact to give rise to the multiple genetic alterations found in human cancers. HCAs are produced under routine cooking conditions (6,13,14) so that total avoidance of exposure is almost impossible. However, reduction of exposure levels as much as possible is advisable. Knowledge of how to minimize the formation of HCAs and to suppress their carcinogenicity by other dietary factors is clearly quite important for cancer prevention. Measures to lessen HCA exposure must be simple and realistic to be acceptable to the general public (13). In the case of endogenous carcinogens, such as the nitroso compounds and APNH reported here, a similar situation can be envisaged. Thus, intake of precursors of endogenous carcinogens including nitrate and nitrosatable compounds should be minimized, and facilitating conditions for formation of endogenous carcinogens in the human body, such as inflammation, should be promptly treated. Moreover, increased intake of anti-oxidants through foodstuffs to prevent the formation of endogenous carcinogens is to be recommended.

At the same time, the formation of environmental mutagens/carcinogens generated as outcomes of industrial activity could be reduced by improvement of technology. PBTA derivatives are thought to be formed from azo compounds through reduction and subsequent chlorination in the processes of discharging and bleaching in textile dyeing factories and also in disinfection process in sewage plants (54). Optimization of discharging and bleaching in textile dyeing factories and usage of alternative azo compounds might be useful and efficient methods to reduce the formation of PBTAs.

There is now abundant evidence that quitting the smoking habit results in a decrease in the risk of lung cancer (73). Infection by hepatitis B and C viruses can be controlled by vaccination and improvement of hygienic conditions, and thereby hepatocellular carcinoma incidences have been shown to remarkably decline (74,75). Several epidemiological investigations have suggested that a positive correlation exists between fat intake and colon cancer incidence, and avoiding overweight and taking regular physical activity are reported to be preventive factors (76,77). These are good examples to show the importance and usefulness of cancer prevention. Identification of environmental mutagens and carcinogens and endogenous mutagens and carcinogens, and elucidation of mechanisms of carcinogenesis provide useful data for effective measures to reduce cancer development. Clearly, knowledge of nutrition and individual dietary factors can also contribute to suppression of carcinogenesis. Environmental carcinogens including food carcinogens, and nutritional conditions continue to be most challenging subjects for cancer control.

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CHEMICAL CONFIRMATION OF THE STRUCTURE OF A MUTAGENIC AMINOPHENYLNORHARMAN, 9-(4'-AMINOPHENYL)-9*H*-PYRIDO[3,4-*b*]INDOLE : AN AUTHENTIC SYNTHESIS OF 9-(4'-NITROPHENYL)-9*H*- PYRIDO[3,4-*b*]INDOLE AS ITS RELAY COMPOUND

Yasuoki Murakami,[a]* Nobuyuki Imai,[a] Tsuyoshi Miura,[a] Takashi Sugimura,[b] Keiji Wakabayashi,[b] Yukari Totsuka,[b] Noriyasu Hada,[c] Yuusaku Yokoyama,[d] Hideharu Suzuki,[d] and Katsuyoshi Mitsunaga[d]

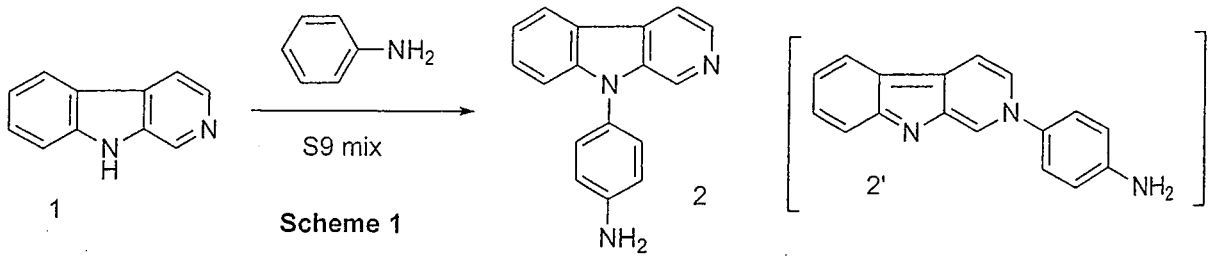
[a]Chiba Institute of Science, Faculty of Pharmacy, 15-8, Shiomicho, Choshi, Chiba, 288-0025, Japan; [b]National Cancer Center Research Institute; [c]Keio University, Faculty of Pharmacy; [d]Toho University, Faculty of Pharmaceutical Sciences.

[a]E-mail:ymurakami@cis.ac.jp

Abstract – 9-(4'-Aminophenyl)-9*H*-pyrido[3,4-*b*]indole **2** is a mutagenic compound produced by non-mutagenic norharman **1** and aniline in the presence of S9 mix. 9-(4'-Nitrophenyl)-9*H*-pyrido[3,4-*b*]indole **4**, the relay compound for synthesis of **2**, was synthesized starting from ethyl indole-2-aldehyde **12** via initial *N*-(4-nitro)phenylation of the indole nucleus, elongation of the 2-aldehyde substituent, and then construction of the pyridine nucleus in order to ensure the nitrogen substitution in **2**.

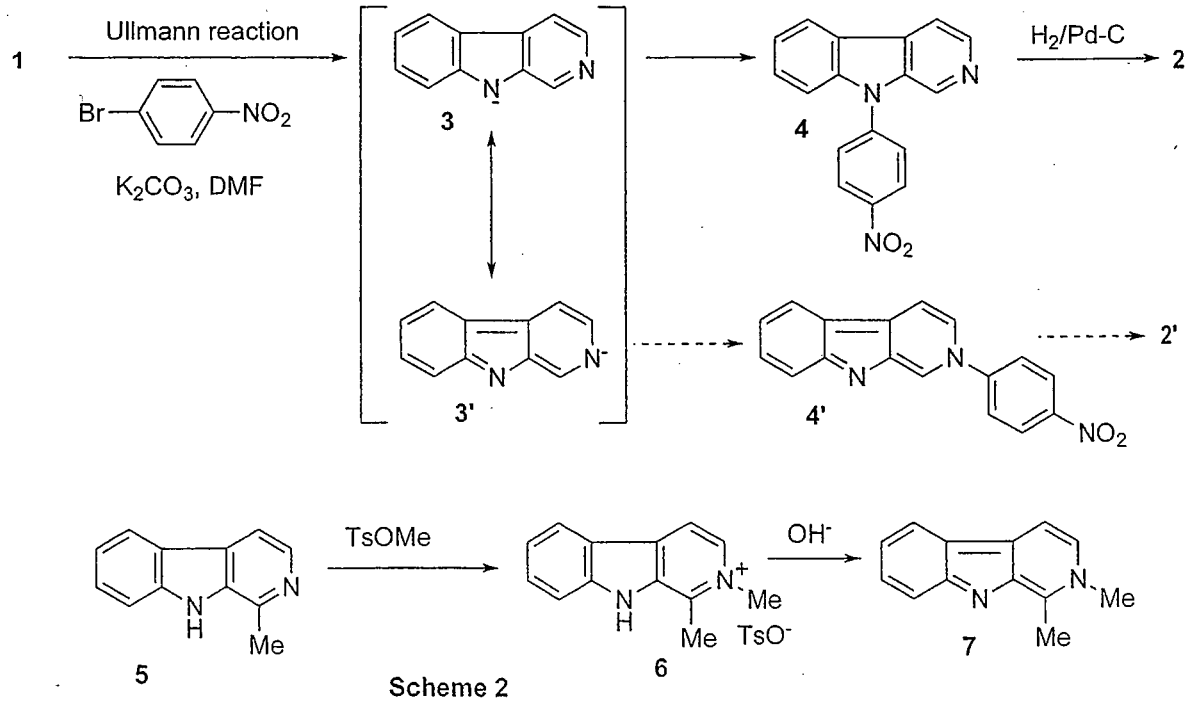
INTRODUCTION

Sugimura et al.[1] reported that norharman **1** (9*H*-pyrido[3,4-*b*]indole, β-carboline) itself is not mutagenic to *Salmonella* strains, but becomes mutagenic to *S. typhimurium* TA98 and YG1024 with S9 mix in the presence of non-mutagenic aromatic amines such as aniline and *o*-toluidine. In a



Scheme 1

subsequent report[2] they isolated mutagenic compound **2** produced by the reaction between norharman and aniline with S9 mix (Scheme 1). In order to elucidate the structure, one of the potential structures, 9-(4'-aminophenyl)-9*H*-pyrido[3,4-b]indole **2**, was synthesized[2] via Ullmann reaction of norharman **1** with 4-bromonitrobenzene, followed by catalytic hydrogenation. The synthetic sample was identical to the natural one and the spectral data of the product supported the structure of **2** but not **2'**. The synthetic strategy was based on the fact that Ullmann reaction of indoles with aryl halides proceeded at its NH position.[3] However, if the reaction occurs on the pyridine nitrogen of **1** via its basicity or another resonance structure **3'**, the product should be compound **4'** (Scheme 1 and 2), whose structure would be much more unstable than the structure **4**, as it has neither benzene, indole, nor pyridine aromaticity any longer. Thus, such a compound is thought to be difficult to produce. On the other hand, it was recently reported[4] that N_A-methylammonium harman **6** derived from harman **5** was basified to yield the compound **7**, whose skeleton is the same as those of **2'** and **4'** (Scheme 2). In this paper we report the authentic synthesis of the relay compound **4** in order to ensure the nitrogen substitution of the substituted phenyl group in **2**.

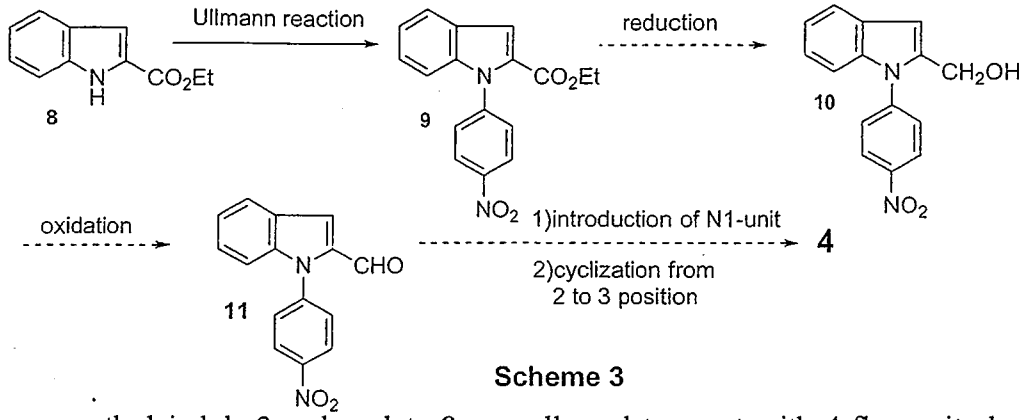


Scheme 2

RESULTS AND DISCUSSION

The synthetic strategy for the synthesis of the relay compound 4 was designed as shown in Scheme 3. The key point is the initial (4-nitro)phenylation at the 1-nitrogen position in the indole nucleus, followed by elongation of the 2-substituent and cyclization to form the pyridine nucleus.

The usual construction of the pyridine ring in the indole nucleus for synthesis of the 9*H*-pyrido[3,4-*b*]indole nucleus is cyclization of the 3-substituent of the tryptamine derivative to the 2-position of the indole nucleus as seen in the Bischler-Napieralski reaction, Pictet-Spengler reaction and so on. On the other hand, there are few methods for cyclization of the 2-substituent to the 3-position of the indole nucleus. Several years ago we developed a method for 9*H*-pyrido[3,4-*b*]indole synthesis of the latter type in the course of the synthetic study of 4-oxo-β-carboline.[5] We applied this method in the present strategy.

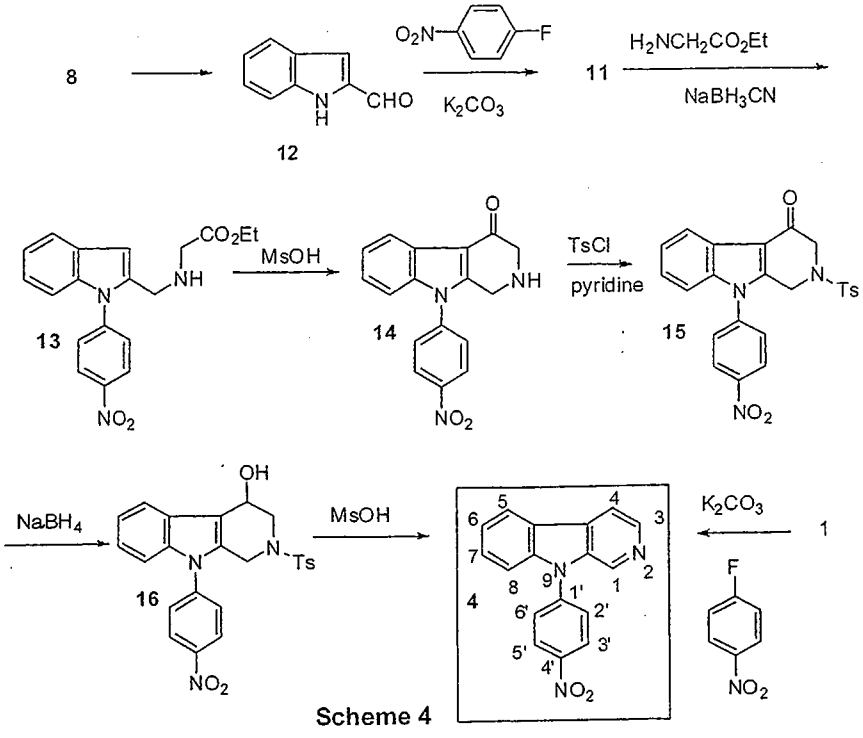


Scheme 3

For this purpose, ethyl indole-2-carboxylate **8** was allowed to react with 4-fluoronitrobenzene to give ethyl *N*-(4'-nitrophenyl)indole-2-carboxylate **9**. However, the reduction of ester carbonyl of **9** with $LiAlH_4$ was not successful (Scheme 3). Thus, the reaction scheme to prepare the aldehyde **11** had to be changed. The synthetic route was changed as in Scheme 4.

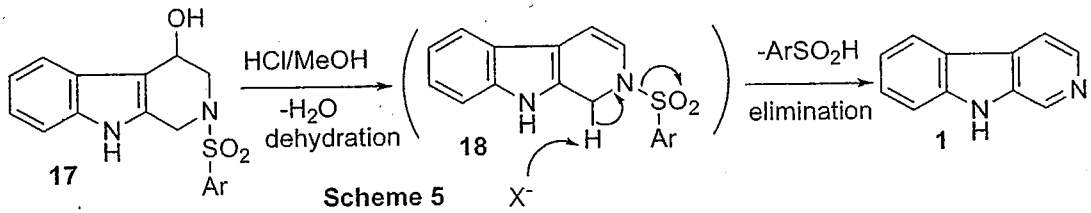
The first *N*-(4-nitro)phenylation of indole-2-carboxaldehyde[5] **12** prepared from **8**, which we feared to proceed with difficulty due to the sensitive reactivity of aldehyde functionality, went much better than expected (51% yield). The *N*-(4'-nitrophenyl)indole-2-carboxaldehyde **11** thus obtained was allowed to react with ethyl aminoacetate and then sodium cyanoborohydride to give the *N*-indolic aminoacetate **13**. The cyclization of **13** with methanesulfonic acid gave the cyclized amino ketone **14**. The aminoketone **14** was treated with tosyl chloride in the presence of pyridine to give the corresponding tosylamide **15** in good yield. The subsequent process of cyclic amino ketone resembling **14** to the target 9*H*-pyrido[3,4-*b*]indole nucleus has already been developed.[6]

The reduction of the ketone of **15** to the hydroxyl group with a large excess amount of sodium borohydride proceeded to give the alcohol **16** in good yield. The last and important dehydration and aromatization processes (two successive β-eliminations) were examined for the present reaction.



Scheme 4

The reaction[6] involved dehydration of the alcohol and β-elimination process around the sulfonyl group with HCl in MeOH, as shown in Scheme 5. In the present case the reaction did not proceed well with HCl in MeOH in several trials, probably due to its insolubility.



Scheme 5

After several acidic conditions were tried, the alcohol **16** was allowed to react with methanesulfonic acid. The target compound **4** was finally obtained from the basic layer in this reaction (20% yield). The product was identified with the already[2] and freshly synthesized sample directly via the Ullmann reaction from **1** and 4-bromo-(or 4-fluoro)nitrobenzene in the presence of K_2CO_3. It was proved that the Ullmann reaction of **1** proceeded at the indolic NH position even on the 9*H*-pyrido[3,4-*b*]indole nucleus. It is worth noting that the Ullmann reaction of 1 with 4-fluoronitrobenzene without K_2CO_3 did not proceed at all. This means that the formation of nitrogen anion is necessary for Ullmann reaction of indole and pyridine nitrogen cannot take Ullmann reaction directly. Thus, the structure **2** was chemically determined. Using this scheme, it may be possible to develop a new strategy for 9*H*-pyrido[3,4-*b*]indole synthesis that involves cyclication of the 2-substituent toward the 3-position of the indole skeleton.

EXPERIMENTAL

All melting points were measured on a hot stage micro-melting points apparatus (Yanagimoto) and are uncorrected. Elemental analyses were conducted with a Yanaco CHN CORDER MT-6. The ^{1}H-NMR spectra were measured with a Bruker Ultrashield^{TM} 400 Plus (400MHz) spectrometer. Deuteriochloroform was used as the solvent with tetramethylsilane as an internal reference. MS spectra were measured on JEOL JMS-GC-mate II and JEOL JMS-600H spectrometers. IR spectra were recorded on a Shimadzu FTIR-8400S spectrometer. For column chromatography, Silica gel 60 (70-230 mesh ASTM; Merck) was used.

1-(4'-Nitrophenyl)indole-2-carboxaldehyde 11

A solution of indole-2-carboxaldehyde[5] **12** (1.80 g, 12.4 mmol), 4-fluoronitrobenzene (5.19 g, 36.8 mmol), and powdered anhydrous K_2CO_3 (5.14 g, 37.2 mmol) in anhydrous DMF (27 mL) was heated with stirring at 100 °C for 1.5 h. The reaction mixture was poured onto water (150 mL), and extracted with AcOEt. The organic layer was washed with water, dried over $MgSO_4$, and evaporated *in vacuo* to dryness. The residue (6.38 g) was chromatographed over SiO_2 with toluene as eluent to give the target compound **11** (1.70 g, 51%). Recrystallization of a part of the compound from a mixture of AcOEt and hexane gave pale yellow columns, mp 170-172 °C. *Anal.* Calcd for $C_{15}H_{10}N_2O_3$: C, 67.67; H, 3.79; N, 10.52. Found: C, 67.98; H, 3.95; N, 10.43. MS ($C_{15}H_{10}N_2O_3$): *m/z*266 (M^+). IR ν max(KBr)cm^{-1}: 1683 (sh), 1672 (CO). ^{1}H-NMR ($CDCl_3$) δ : 7.23-7.58 (6H, m, $C_{3,5,6,7,2',6'}$-H), 7.83 (2H, m, C_4-H), 8.41 (2H, d, *J*=8.0 Hz, $C_{3'}$,$C_{5'}$-H), 9.89 (1H, s, CHO).

Ethyl [1-(4'-Nitrophenyl)indole-2-ylmethyl]aminoacetate 13

To a muddy solution of 1-(4'-nitrophenyl)indole-2-carboxaldehyde **11** (724 mg, 2.77 mmol) and ethyl aminoacetate hydrochloride (1.12 g, 8.16 mmol) in ethanol (30 mL) was added triethylamine (1.17 mL, 8.16 mmol) and $NaBH_3CN$ (685 mg, 10.9 mmol) successively with stirring under ice-cooling. The reaction mixture (muddy state) was stirred under ice-cooling for 15 min and then at rt for an additional 3 h. Then, the reaction mixture was poured onto water and extracted with AcOEt. The organic layer was washed with brine, dried over $MgSO_4$ and evaporated *in vacuo* to dryness to give a pale yellow residue. The crude products were chromatographed over SiO_2. Elution with toluene, followed by toluene-AcOEt (10:1), gave the target compound **13** (713 mg, 74%) as a pale yellow oil. MS ($C_{19}H_{19}N_3O_4$): *m/z* 353 (M^+). HRMS: Calcd for $C_{19}H_{19}N_3O_4$, 353.1376; Found, 353.1378. IR ν max($CHCl_3$)cm^{-1}: 3684, 3620 (NH), 1734 (C=O). ^{1}H-NMR δ :1.23 (3H, t, *J*=8.0 Hz, -$CH_2\underline{CH_3}$), 3.41 (2H, s, -CH_2NH-), 3.94 (2H, s, -NCH_2CO-), 4.12 (2H, *J*=8.0 Hz, -O$\underline{CH_2}CH_3$), 6.75 (1H, s, C_3-H), 7.18-7.26 (3H, m, $C_{5,6,7}$-H), 7.63 (1H, m, C_4-H), 7.73 (2H, *J*=8.0 Hz, $C_{2',6'}$-H), 8.41 (2H, $C_{3',5'}$-H).

fluoronitrobenzene (417 mg, 3 mmol), and powdered anhydrous K_2CO_3 (304 mg, 2.2 mmol). The whole was heated at 100 °C under stirring for 14.5 h. The reaction mixture was poured onto water, and extracted with AcOEt. The organic layer was washed with water, dried over $MgSO_4$, and evaporated to dryness *in vacuo*. The residue (702 mg) was chromatographed over SiO_2 and eluted with toluene to give the target compound **9** (266 mg, 54%). A part of this compound was recrystallized from AcOEt-hexane to give pale yellow plates, mp 133-135 °C. *Anal.* Calcd for $C_{17}H_{14}N_2O_4$: C, 65.80; H, 4.55; N, 9.03. Found: C, 65.99; H, 4.64; N, 8.76. MS ($C_{17}H_{14}N_2O_4$): 310 (M^+). IR ν max(KBr)cm^{-1}: no NH, 1704 (CO). ^{1}H-NMR δ : 1.29 (3H, t, *J*=8.0 Hz, -$CH_2C\underline{H}_3$), 4.25 (2H, t, *J*=8.0 Hz, -$OC\underline{H}_2CH_3$), 7.13 (1H, d, *J*=1.5 Hz, C_3-H), 7.23-7.36 (3H, m, indolic Hs), 7.52-7.56 (3H, m, $C_{2'}$,$C_{6'}$,an indolic H), 7.76 (1H, d, *J*=9.0 Hz, C_4-H), 8.40 (2H, d, *J*=9.0 Hz, $C_{3'}$,$C_{5'}$-H).

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Dose-dependent alterations in gene expression in mouse liver induced by diethylnitrosamine and ethylnitrosourea and determined by quantitative real-time PCR☆

Takashi Watanabe[a], Gotaro Tanaka[b], Shuichi Hamada[c], Chiaki Namiki[d], Takayoshi Suzuki[e], Madoka Nakajima[f], Chie Furihata[a,*]

[a] Functional Genomics Laboratory, School of Science and Engineering, Aoyama Gakuin University, Fuchinobe 5-10-1, Sagamihara, Kanagawa 229-0006, Japan
[b] Tokushima Research Center, Taiho Pharmaceutical Co. Ltd., Hiraishiebisuno 224-2, Kawauchichou, Tokushima, Tokushima 771-0194, Japan
[c] Genetic Toxicology Group, Toxicology Division II, Kashima Laboratory, Mitsubishi Chemical Safety Institute Ltd., Sunayama 14, Kamisu-shi, Ibaraki 314-0255, Japan
[d] Central Research Laboratory, SSP Co. Ltd., Nanpeidai 1143, Narita, Chiba 286-8511, Japan
[e] Division of Cellular & Gene Therapy Products, National Institute of Health Sciences, Kamiyoga 1-18-1 Setagaya-ku, Tokyo 158-8501, Japan
[f] Genetic Toxicology Group, Biosafety Research Center, Foods, Drugs, and Pesticides, Shioshinden 582-2, Fukude-cho, Iwata-gun, Shizuoka 437-1213, Japan

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ABSTRACT

We examined the dose-dependency of gene expression changes for 51 genes in mouse liver treated with two *N*-nitroso genotoxic hepatocarcinogens, diethylnitrosamine (DEN) and ethylnitrosourea (ENU) by quantitative real-time PCR (qPCR). DEN (3, 9, 27 and 80 mg/kg bw) or ENU (6, 17, 50 and 150 mg/kg bw) was injected intraperitoneally into groups of five male 9-week-old B6C3F$_1$ mice and the livers were dissected after 4 h and 28 days. Total RNA from pooled livers was reverse-transcribed to cDNA and the amount of each gene was quantified by qPCR. Results were analyzed by hierarchical and *k*-means clustering and ingenuity pathway analysis (IPA). The most characteristic result was a similar dose-dependency of gene expression changes with DEN and ENU. Twenty-one genes exhibited a distinct dose-dependent increase in expression at 4 h for both carcinogens [*Bax*, *Btg2*, *Ccng1*, *Cdkn1a*, *Cyp4a10*, *Cyp21a1*, *Fos*, *Gadd45b*, *Gdf15*, *Hmox1*, *Hspb1*, *Isg20l1*, *Jun*, *Mbd1*, *Mdm2*, *Myc*, *Net1*, *Plk2*, *Ppp1r3c*, *Rcan1* and *Tubb2c*], although the increase in gene expression due to ENU was generally weaker than that due to DEN. Only *Gdf15* showed a dose-dependent increase in expression at 28 days for both carcinogens. The differences between DEN and ENU were in the expression of additional genes (7 for DEN and 8 for ENU). IPA extracted five gene networks: Network-1 included genes related to cancer and cell cycle arrest and associated with *Bax*, *Btg2*, *Ccng1*, *Cdkn1a*, *Gadd45b*, *Gdf15*, *Hspb1*, *Mdm2* and *Plk2* and Network-2 was related to DNA replication, recombination, repair and cell death and associated with *Cyp21a1*, *Gdf15*, *Ppp1r3c*, *Rcan1* and *Tubb2c*. The present results show a distinct dose-dependency of gene expression changes induced by DEN and ENU. These changes were associated with cancer, cell cycle arrest, DNA replication, recombination, repair and cell death and were seen not only at 4 h but also, for some, at 28 days after administration.

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1. Introduction

Diethylnitrosamine (DEN) and ethylnitrosourea (ENU) are potent genotoxic *N*-nitroso carcinogens that induce hepatocellular carcinomas in mouse liver [1,2]. It has been reported that after its metabolic biotransformation, DEN produces the promutagenic adducts O^6-ethylguanine (O^6-EtG) and O^4- and O^2-ethylthymine and that O^4-ethylthymine may be responsible for the initiation of hepatocellular carcinomas in rats [3]. ENU, which is a direct-ethylating agent, forms several major adducts upon reaction with DNA, of which O^6-EtG, O^4- and O^2-ethylthymine and N^3-ethylthymine have been implicated in mutagenic lesions [4]. Suzuki et al. have reported that mutagenic activity by DEN and ENU was clearly detected with the *lacZ* mutation assay in mouse liver at 7 days [5]. Mientjes et al. have reported that the O^6-EtG levels increased as early as 1.5 h after treatment, whereas at 3 days more than 90% of the lesions had been removed from the DNA in the livers of DEN- and ENU-treated mice, based on *lacZ* transgenic mice [6]. After this period, however, with the bulk of O^6-EtG removed, the induction of *lacZ* mutations was observed at 3 days and continued to increase for some weeks.

☆ This work was a JEMS/MMS/Toxicogenomics group collaborative study.

* Corresponding author at: Department of Chemistry and Biological Science, School of Science and Engineering, Aoyama Gakuin University, 5-10-1 Fuchinobe, Sagamihara, Kanagawa 229-8558, Japan. Tel.: +42 759 6233; fax: +42 759 6511.
E-mail address: chiefurihata@gmail.com (C. Furihata).

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Previously, Waring et al. showed by DNA microarray that a number of genes are up-regulated and down-regulated in rat liver, with rats dosed daily with DEN for 3 days and euthanized on the 4th day [7]. Genes up-regulated by DEN included genes related to growth arrest and DNA damage, such as *Bax*, *Ccnd1*, *Ccng1*, *Cdkn1a/p21*, *Gadd45* and *Jun*. However, no studies have focused on either the DNA damaging time of 4 h or the mutation fixing time of 28 days in DEN-treated mouse or rat liver. Although it has been reported that ENU induced expression of *Bax*, *Crp*, *Cyp2a*, *Gstm2*, *Icam1*, *Mig*, and *Mt2* mRNA in mouse liver, little is known about differential gene expression in ENU-exposed rodent liver [8].

Quantitative real-time PCR (qPCR) is an alternative technology for toxicogenomics [9]. qPCR is a highly regarded and reliable quantitative method but analysis of a large number of genes may be lengthy. It is impractical to examine a great number of genes with qPCR. Therefore, we selected 51 candidate genes (Table 1) based on our previous results using the Affymetrix GeneChip Mu74AV2 and original DNA microarray to determined the effects of DEN, dimethylnitrosamine, dipropylnitrosamine, ENU, *o*-aminoazotoluene, 7,12-dimethylbenz[*a*]anthracene, dibenzo[*a*,*l*]pyrene, phenobarbital and ethanol exposure in mouse liver for 4 and 20 h and 14 and 28 days in our JEMS/MMS/Toxicogenomics group collaborative study; results were reported in part [10]. We examined gene expression changes at an early time after administration, as we were interested in whether toxicogenomics was useful for carcinogen screening. In the previous study, using a single dose for each chemical, gene expression changes in number and degree were observed to peak at 4 h after administration. It is known that genotoxic *N*-nitroso carcinogens induce DNA damage and repair in a matter of a few hours after their administration; DNA adducts [6], DNA strand-breaks [11], unscheduled DNA synthesis [12] and other lesions have been reported. It is also known that mutations are observed in transgenic mouse liver 28 days after genotoxic *N*-nitroso carcinogen administration [5,6]. However, related gene expression changes at these time points have not yet been fully elucidated.

Table 1
Fifty-one genes examined in the present study.

No.	Symbol	Gene name	Accession number
1	*Bax*	Bcl2-associated X protein	NM_007527
2	*Bcl2*	B-cell leukemia/lymphoma 2	NM_009741
3	*Btg2*	B-cell translocation gene 2, anti-proliferative	NM_007570
4	*Casp1*	IL-1B converting enzyme; interleukin 1 beta-converting enzyme	NM_009807
5	*Ccnf*	Cyclin F	NM_007634
6	*Ccng1*	Cyclin G1	NM_009831
7	*Ccng2*	Cyclin G2	NM_007635
8	*Cdkn1a (p21)*	Cyclin-dependent kinase inhibitor 1A (P21)	NM_007669
9	*Cyp1a1*	Cytochrome P450, family 1, subfamily a, polypeptide 1	NM_009992
10	*Cyp1a2*	Cytochrome P450, family 1, subfamily a, polypeptide 2	NM_009993
11	*Cyp4a10*	Cytochrome P450, family 4, subfamily a, polypeptide 10	NM_010011
12	*Cyp21a1*	Cytochrome P450, family 21, subfamily a, polypeptide 1	NM_009995
13	*Dpyd*	Dihydropyrimidine dehydrogenase	NM_170778
14	*Egfr*	Epidermal growth factor receptor	NM_207655
15	*Ephx1*	Epoxide hydrolase 1, microsomal	NM_010145
16	*Fabp5*	Fatty acid binding protein 5, epidermal	NM_010634
17	*Fos*	FBJ osteosarcoma oncogene	NM_010234
18	*Gadd45b*	Growth arrest and DNA-damage-inducible 45 beta	NM_008655
19	*Gadd45g*	Growth arrest and DNA-damage-inducible 45 gamma	NM_011817
20	*Gapdh*	Glyceraldehyde-3-phosphate dehydrogenase	NM_008084
21	*Gdf15*	Growth differentiation factor 15	NM_011819
22	*Glul*	Glutamate-ammonia ligase (glutamine synthetase)	NM_008131
23	*Gstk1*	Glutathione S-transferase kappa 1	NM_029555
24	*Gyk*	Glycerol kinase	NM_212444
25	*Hist1h1c*	H1 histone family, member 2	NM_015786
26	*Hspa1b (Hsp70)*	Heat shock protein 1B	NM_010478
27	*Hspb1*	Heat shock protein 1	NM_013560
28	*Hspb2 (Hsp27)*	Heat shock protein 2	NM_024441
29	*Hmox1*	Heme oxygenase (decycling) 1	NM_010442
30	*Hprt1*	Hypoxanthine guanine phosphoribosyl transferase 1	NM_013556
31	*Igfbp1*	Insulin-like growth factor binding protein 1	NM_008341
32	*Isg20l1*	Interferon stimulated exonuclease gene 20-like 1	NM_026531
33	*Jun*	Jun oncogene	NM_010591
34	*Kras*	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog	NM_021284
35	*Lig3*	Ligase III, DNA, ATP-dependent	NM_010716
36	*Lrp1*	Low density lipoprotein receptor-related protein 1	NM_008512
37	*Mbd1*	Methyl-CpG binding domain protein 1	NM_013594
38	*Mdm2*	Transformed mouse 3T3 cell double minute 2	NM_010786
39	*Myc*	Myelocytomatosis oncogene	NM_010849
40	*Net1*	Neuroepithelial cell transforming gene 1	NM_019671
41	*Pdgfb*	Platelet-derived growth factor, B polypeptide	NM_011057
42	*Plk2*	Polo-like kinase 2; serum-inducible kinase	NM_152804
43	*Pml*	Promyelocytic leukemia	NM_008884
44	*Pmm1*	Phosphomannomutase 1	NM_013872
45	*Ppp1r3c*	Protein phosphatase 1, regulatory (inhibitor) subunit 3C	NM_016854
46	*Rad52*	RAD52 homolog (S. cerevisiae)	NM_011236
47	*Rcan1(Dscr1)*	Regulator of calcineurin 1	NM_019466
48	*Trp53*	Transformation related protein 53	NM_011640
49	*Tubb2c*	Tubulin, beta 2c	NM_146116
50	*Ube2e1 (UbcM3)*	Ubiquitin-conjugating enzyme E2E 1, UBC4/5 homolog (yeast)	NM_009455
51	*Ung*	Uracil-DNA glycosylase	NM_011677

In this paper, we report our studies of gene expression changes in B6C3F$_1$ mouse liver induced by multiple doses of two typical alkylating agents, DEN and ENU. We investigated the dose-dependency of gene expression changes at two different time points: 4 h, characterized by the production of many DNA lesions, and 28 days, characterized by fixing of mutations [6]. If we could show dose-dependency in gene expression changes at 4 h, we could clarify key genes related to DNA lesions and subsequent various phenomena in liver cells induced by DEN and ENU. If we could show the dose-dependency in gene expression changes at 28 days, we could clarify key genes related to effects of mutations and subsequent changes that may be causal for carcinogenesis. Our purpose is to determine biological cell responses induced by DEN and ENU by examining the dose-dependency at these two time points.

In addition, we examined gene networks using IPA to elucidate interactions between genes with altered expression.

2. Materials and methods

2.1. Animal treatment

Male B6C3F$_1$ mice were obtained at 8 weeks of age from Charles River Japan, Inc. (Yokohama, Japan). They were kept in plastic cages on wood chips as bedding and given food (Oriental MF, Oriental Yeast Co., Tokyo) and water *ad libitum* in an air-conditioned room [12 h light (7 a.m. to 7 p.m.), 12 h dark; 23 ± 2 °C; 55 ± 5% humidity]. All animal experiments were conducted in accordance with the NIH Guide for Care and Use of Laboratory Animals and approved by the Animal Care and Use Committee at the Mitsubishi Chemical Safety Institute Ltd.

Mice at 9 weeks of age were injected intraperitoneally (i.p.) with DEN (3, 9, 27 and 80 mg/kg bw; Wako Pure Chem. Ind. Ltd., Osaka, Japan; CAS 55-18-5) dissolved in sterile water or ENU (6, 17, 50 and 150 mg/kg bw; Wako Pure Chem. Ind. Ltd., Osaka, Japan; CAS 759-73-9) dissolved in sterile water. Control animals for the DEN- and ENU-treated groups received sterile water. At 4 h and 28 days after treatment, animals were sacrificed after which the liver was collected, frozen on dry ice, and stored at −80 °C until use.

2.2. RNA isolation and relative quantification by real-time PCR

To isolate total RNA, approximately 150 mg from each liver (main lobe) was placed into TRIzol reagent (Invitrogen Corp., Carlsbad, CA, USA) and immediately homogenized using a Potter homogenizer. The samples were further homogenized with a 1 ml syringe and 18 gauge needle. Finally, total RNA was purified using an ethanol precipitation method. Complementary DNA (cDNA) was yielded from total RNA using the SuperScript First strand synthesis system for RT-PCR kit (Invitrogen Corp.).

qPCR amplifications were performed in triplicate using the SYBR Green I assay in an Opticon II (MJ Research, Inc., Waltham, MA, USA). The reactions were carried out in a 96-well plate in 20-μl reactions containing 2× SYBR Green Master Mix (Applied Biosystems, Lincoln Centre Drive Foster City, CA, USA), 2 pmol each of forward and reverse primer, and a cDNA template corresponding to 10 ng total RNA. Each primer sequence and Ct value are shown in Table 2. We selected 51 genes based on our previous results from the original DNA microarray and Affymetrix GeneChip Mu74AV2 for samples after treatment of DEN, dimethylnitrosamine, dipropylnitrosamine, ENU, o-aminoazotoluene, 7,12-dimethylbenz[*a*]anthracene, dibenzo[*a,l*]pyrene, phenobarbital and ethanol in our JEMS/MMS/Toxicogenomics group collaborative study. *Gapdh* and *Hprt1* were selected as housekeeping genes. SYBR Green PCR conditions were 95 °C for 10 min, followed by 95 °C for 10 s, 58 °C for 50 s and 72 °C for 20 s, for 45 cycles. In each assay a standard curve was determined concurrently with examined samples. In the preliminary experiment the highest group was selected for each gene and was used as the standard sample in the subsequent assay. In each standard curve determination, there were six dilution series of standard samples, diluted up to 1/5, 1/25, 1/125, 1/625 and 1/3125 of the selected standard liver cDNA for each gene. Finally, relative quantitative values of each sample were determined with 1/25 diluted cDNA and were normalized with those of the *Gapdh* genes. Relative *Gapdh* expression levels of experimental groups are presented in Fig. 1.

2.3. Data analysis and clustering algorithm

For the cluster analysis program, we performed a logarithmic ($\log_2$) transformation of the data to stabilize the variance and the gene expression profile of each DEN- and ENU-treated sample, normalized to the median gene expression level for the entire sample set. Both hierarchical and *k*-means clustering were performed using GENESIS software (http://genome.tugraz.at/) [13] for each data set at 4 h and 28 days separately. Gene groups were presented automatically by hierarchical clustering. Four clusters were set up initially in *k*-means clustering based on hierarchical clustering results. Genes which belonged to dose–response groups by both clustering methods were defined as dose–response genes. Furthermore, genes which showed less than a 0.5-fold decrease dose-dependently were evaluated as decrease genes by expression pattern because the decrease genes were few and could not be extracted using both clustering methods.

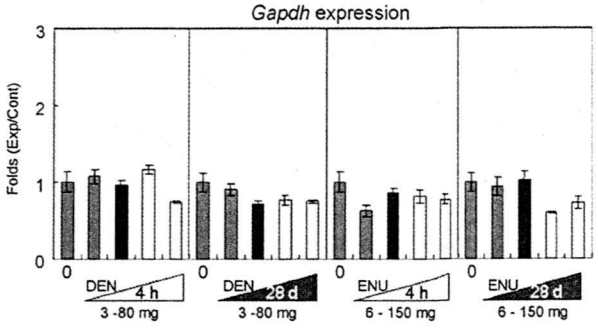

Fig. 1. Relative expression of *Gapdh*. DEN (0–80 mg/kg bw) and ENU (0–150 mg/kg bw) were given to 9-week-old mice (five per group). Total RNA was extracted from pooled liver and reverse-transcribed to cDNA. *Gapdh* expression was determined by qPCR in triplicate assays. Results are shown as mean ± S.D.

The color displays given in Fig. 2 show the $\log_2$ (expression ratio) as (1) red when the treatment sample is up-regulated relative to the control sample, (2) blue when the treatment sample is down-regulated relative to the control sample and (3) white when the $\log_2$ (expression ratio) is close to zero.

2.4. Pathway analysis

Numerical experimental data at 4 h and 28 days after DEN or ENU treatment were separately analyzed by ingenuity pathway analysis (IPA) Software-Complete Pathways Database. These data were generated through the use of IPA, a web-delivered application (www.Ingenuity.com) that enables the visualization and analysis of biologically relevant networks to discover, visualize, and explore therapeutically relevant networks. IPA information was extracted by experts from the full text of the scientific literature, including information about genes, drugs, chemicals, cellular and disease processes, and signaling and metabolic pathways.

Expression data sets containing gene identifiers (Entrez gene identifiers) and their corresponding expression values as fold changes were uploaded as a tab-delimited text file. Each gene identifier was mapped to its corresponding gene object in the Ingenuity Pathways Knowledge Base. To start building networks, the application program queries the Ingenuity Pathways Knowledge Base for interactions between focus genes and all other gene objects stored in the knowledge base and generates a set of networks. The program then computes a score for each network according to the fit of the network to the set of focus genes. The score indicates the likelihood of the focus genes in a given network being found together due to random chance. A score of >2 indicates that there is a <1 in 100 chance that the focus genes were assembled randomly into a network due to random chance.

3. Results

3.1. Dose-dependent alteration of gene expression induced by DEN

3.1.1. Clustering analysis for gene expression

Unsupervised hierarchical clustering results are shown in Fig. 2. The changes in gene expression are represented colorimetrically as described in Section 2. The clustering presented four groups (DEN-4 h-Grp-1 to DEN-4 h-Grp-4) and an ungrouped gene 4 h after administration, and three groups (DEN-28 d-Grp-1 to DEN-28 d-Grp-3) and eight ungrouped genes 28 days after administration. As unsupervised hierarchical clustering was performed for 4 h and 28-day samples separately, group member genes were different for 4 h groups and 28-day groups.

At 4 h, all 20 DEN-4 h-Grp-1 genes showed a dose-dependent increase of more than 3–64-fold. Twelve DEN-4 h-Grp-2 genes were suggested to have a gradual dose-dependent increase of less than that for the expression in DEN-4 h-Grp-1. Two DEN-4 h-Grp-4 genes exhibited a dose-dependent decrease of less than 0.3-fold.

Table 2
Primer sequences of 51 genes examined in the study.

No.	Symbol	Left	Right	Ct
1	*Bax*	CCAGGATGCGTCCACCAAGAAG	GGAGTCCGTGTCCACGTCAGC	28
2	*Bcl2*	GATGACTTCTCTCGTCGCTACC	CATCCCTGAAGAGTTCCTCCAC	31
3	*Btg2*	ACGGGAAGAGAACCGACATGC	ATGATCGGTCAGTGCGTCCTG	24
4	*Casp1*	GTCTTGGAGACATCCTGTCAGG	GCATCTGTAGCCTAAATTCTGG	32
5	*Ccnf*	AGCACAAAGCCTTGCCACCATC	AAGCCAGGTGCGTGTCCTTGTC	25
6	*Ccng1*	TGGCCGAGATTTGACCTTCTGG	GTGCTTCAGTTGCCGTGCAGTG	22
7	*Ccng2*	GCCATCAAGCTAGGACTGTTAG	CACTTATCAACTCCATTCCCTG	26
8	*Cdkn1a (p21)*	TCCCGTGGACAGTGAGCAGTTG	CGTCTCCGTGACGAAGTCAAAG	22
9	*Cyp1a1*	TGGCCGATCGGAGGTCTTTC	AAGTGTTCACAGCGGGCGTG	29
10	*Cyp1a2*	GATGCTCTTCGGCTTGGGAAAG	CCATAGTTGGGTGTCAGGTCCAC	20
11	*Cyp4a10*	AGCCACAAGGGCAGTGTTCAGG	CCAAGCGGCCATTGGAAGAAAG	23
12	*Cyp21a1*	TGTGCTGCCCTTAAAGAAGAGTG	TTGAGCATCCCGTTCCCGTTTC	25
13	*Dpyd*	GTGCGGCTAAAGGCTGATGTGG	CCCATGGTTCACTGGTTTGCATG	24
14	*Egfr*	AGAACGCCTTCCACAGCCAC	ACTCTCGGAACTTTGGGCGG	22
15	*Ephx1*	CATTGTCTCCTCCCAGCGCTTC	GGGCATGCAGGATCTCAGAAGG	21
16	*Fabp5*	ACGGTCTGCACCTTCCAAGACG	ACCCGAGTGCAGGTGGCATTG	24
17	*Fos*	GTCGACCTAGGGAGGACCTTAC	CATCTCTGGAAGAGGTGAGGAC	31
18	*Gadd45b*	TGTACGAGGCGGCCAAACTG	TGTCGCAGCAGAACGACTGG	28
19	*Gadd45g*	GGAAAGCACAGCCAGGATGCAG	ATTCAGGACTTTGGCGGACTCG	26
20	*Gapdh*	GCTCTCAATGACAACTTTGTCAAG	CTTCCTTGGAGGCCATGTAGGC	22
21	*Gdf15*	AGCTGGAACTGCGCTTACGGG	CTCCAGCCCAAGTCTTCAAGAG	28
22	*Glul*	GGAATGGAGCAGGAATATACTC	ACCGCAGTAATACCGGCCTTG	22
23	*Gstk1*	CGTACTCCTGGCTGGGGCTTTG	CAGGTGGTTGGTTGCCGCTGTC	24
24	*Gyk*	GCCTGAAACAACTGCACTAGGC	CACAGCTTTCTTCCATGTGGAG	27
25	*Hist1h1c*	CGAGCTCATCACCAAGGCTGTG	CCCTTGCTCACCAGGCTCTTC	26
26	*Hspa1b (Hsp70)*	GACAAGTCGGAGAACGTGCAG	CGAGTAGGTGGTGAAGGTCTG	25
27	*Hspb1*	CGGTGCTTCACCCGGAAATAC	GCTGACTGCGTGACTGCTTTGG	25
28	*Hspb2 (Hsp27)*	CTCACAGTGAAGACCAAGGAAG	GGATAGGGAAGAGGACACTAGG	26
29	*Hmox1*	AAGACCGCCTTCCTGCTCAAC	CGAAGTGACGCCATCTGTGAGG	28
30	*Hprt1*	CTTGCTCGAGATGTCATGAAGGAG	TAATCCAGCAGGTCAGCAAAGAAC	26
31	*Igfbp1*	GATCAGCCCATCCTGTGGAACG	TTCTCGTTGGCAGGGCTCCTTC	24
32	*Isg20l1*	TTGAAGGGCAAGGTGGTGGTG	GAGCAGGTTTGGGACATAAGTG	24
33	*Jun*	GCCAAGAACTCGGACCTTCTC	AGTGGTGATGTGCCCATTGCTG	23
34	*Kras*	GGCAAGAGCGCCTTGACGATAC	TGGTCCCTCATTGCACTGTACTCC	28
35	*Lig3*	TGCGGCTCTACTTGCCACCTTC	CATGTGTGGCTGAGCCCATGTC	27
36	*Lrp1*	GGGCCATGAATGTGGAAATTGG	GTGGCATACACTGGGTTGGTG	22
37	*Mbd1*	GGATCCTGACACTCAAGAATGG	GTTTGGGCTAACACAGGAAGAG	23
38	*Mdm2*	TTGATCCGAGCCTGGGTCTGTG	AAGATCCTGATGCGAGGGCGTC	27
39	*Myc*	B5,6TCAGCAACAACCGCAAGTGCTC	AAAGCTGCGCTTCAGCTCGTTC	32
40	*Net1*	GACCTCCACGAAGAGTGTGAAG	CTGTACACTGGAGCCACAATCC	27
41	*Pdgfb*	AAGACGCGCACAGAGGTGTTCC	GGCATTGCACATTGCGGTTATTG	33
42	*Plk2*	CTGTTGAGAGCGTCTTCAGTTG	CCATAGTTCACAGTTAAGCAGC	28
43	*Pml*	GGCAAGAAGCGTCCTTACCTTC	GGACAGCAACAGCAGTTCAGTC	28
44	*Pmm1*	TGTCCCGAGGAGGCATGATAAG	CAAAGTCATTCCCGCCAGGAC	30
45	*Ppp1r3c*	TGGAAACCTGACGGAGTGCAG	GCAAGCCTTGGACTGCCAAAG	24
46	*Rad52*	TGACGCCACTCACCAGAGGAAG	GCTGGAAGTACCGCATGCTTGG	30
47	*Rcan1*	GGTCCACGTGTGTGAGAGTG	TGGATGGGTGTGTACTCCGG	24
48	*Trp53*	TTGGACCCTGGCACCTACAATG	GCAGACAGGCTTTGCAGAATGG	26
49	*Tubb2c*	TTGGCAACAGCACCGCTATTC	TCGGACACCAGGTCGTTCATG	23
50	*Ube2e1 (UbcM3)*	AACTGGAGCCCAGCCCTAACC	TGGCCATTCTGTCGTGTTCTGC	24
51	*Ung*	AACCTGAGTGGCCTCGTCTTCC	TCTGCATCCCAGGAACCCTCTG	29

Ct values are those of the highest group in the present experimental condition.

At 28 days, three DEN-28 d-Grp-1 genes showed a dose-dependent increase of more than four-fold. Seventeen DEN-28 d-Grp-2 genes were suggested to have a gradual dose-dependent increase, though less than that for the expression in DEN-28 d-Grp-1. Ungrouped *Igfbp1* showed a dose-dependent decrease of less than 0.3-fold.

Unsupervised *k*-means clustering results are shown in Fig. 3A. Genes were classified into four clusters based on the hierarchical clustering results. Gene expression was classified into four clusters (DEN-4 h-Cluster-1 to DEN-4 h-Cluster-4) 4 h after administration, and four clusters (DEN-28 d-Cluster-1 to DEN-28 d-Cluster-4) 28 days after administration. As unsupervised *k*-means clustering was performed for 4 h and 28-day data separately, cluster member genes were different for 4 h and 28 days.

At 4 h, all 12 DEN-4 h-Cluster-1 genes exhibited a dose-dependent increase of more than eight-fold. Fourteen DEN-4 h-Cluster-2 genes showed a gradual dose-dependent increase as compared to DEN-4 h-Cluster-1 genes. Although *Myc* and *Igfbp1* in DEN-4 h-Cluster-3 had some atypical dose–response, they showed an increase of up to or greater than two-fold, as a whole. Two genes in DEN-4 h-Cluster-4 exhibited a dose-dependent decrease of less than 0.3-fold [*Cyp1a2* and *Glul*]. For 28-day data, 4 DEN-28 d-Cluster-1 genes showed a dose-dependent increase of more than two-fold. *Igfbp1* in DEN-28 d-Cluster-3 showed a dose-dependent decrease of less than 0.3-fold.

Two types of clustering results for the DEN data are summarized as follows. A total of 28 genes showed a dose-dependent increase or decrease at 4 h or 28 days after administration. Twenty-six genes in DEN-4 h-Grp-1 or DEN-4 h-Grp-2 and DEN-4 h-Cluster-1, DEN-4 h-Cluster-2 or DEN-4 h-Cluster-3 showed a dose-dependent increase ranging from 2-fold to more than 64-fold [*Bax*, *Btg2*, *Ccng1*, *Ccng2*, *Cdkn1a*, *Cyp4a10*, *Cyp21a1*, *Fos*, *Gadd45b*, *Gdf15*, *Hspb1*, *Hmox1*, *Hsp27*, *Igfbp1*, *Isg20l1*, *Jun*, *Mbd1*, *Mdm2*, *Myc*, *Net1*, *Plk2*, *Pmm1*, *Ppp1r3c*, *Rad52*, *Rcan1* and *Tubb2c*]. Two genes in DEN-4 h-Grp-4