Formation and stability of 4-(hydroxymethylnitrosamino)-1-(3-pyridyl)-1-butane glucuronide, a stable form of reactive intermediate produced from 4-(methylnitrosamino)-1-(3-pyridyl)-1-butaneone, in mice

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ABSTRACT — 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butaneone (NNK), a tobacco-specific nitrosamine, induced lung tumors in rodents and is likely involved in human lung cancer. 4-(Hydroxymethylnitrosamino)-1-(3-pyridyl)-1-butaneone (HO-methyl NNK) glucuronide, a glucuronide of the reactive intermediate of NNK, has been identified in rats. The aim of this study is to estimate the role of HO-methyl NNK glucuronide in the tumorigenic effects of NNK. We investigated the urinary excretion and tissue distribution of HO-methyl NNK glucuronide in A/J mice, which are susceptible to NNK carcinogenesis, and C57BL/6J mice, which are resistant to NNK carcinogenesis. The cumulative urinary excretion of the HO-methyl NNK glucuronide in the C57BL/6J mice was more than 20 times higher than in the A/J mouse urine. Tissue concentrations of HO-methyl NNK glucuronide were also higher in the C57BL/6J mice than in the A/J mice. Assessment of the stability of HO-methyl NNK glucuronide in liver homogenates at physiological pH conditions showed that more than 60% of the glucuronide remained until 2 hr of incubation. These results suggested that HO-methyl NNK glucuronide is likely to be a detoxified metabolite and could be one reason for differences in the susceptibility to NNK tumorigenesis between the two strains. Once HO-methyl NNK is formed in tissues, C57BL/6J mice have a high ability to form HO-methyl NNK glucuronide so that HO-methyl NNK, the reactive intermediate formed from NNK, is readily excreted in urine as a stable form.

Key words: UDP-glucuronosyltransferase, NNK, Reactive intermediate, Glucuronide, Target organs, β-Glucuronidase

INTRODUCTION

Cigarette smoke contains many chemicals. Of these, more than 250 chemicals are known to be harmful to human health, and more than 60 have been established as carcinogens (U.S. Department of Health and Human Services, 2010; National Toxicology Program, 2005). Among the chemicals suspected of having carcinogenic properties, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butaneone (NNK), which is one of the nitrosamines derived from nicotine, has been classified as a group 1 carcinogen, that is, specifically carcinogenic to humans (IARC, 2007). NNK is also strongly carcinogenic in experimen-
the N-nitroso group of NNK produces the reactive intermediate 4-(hydroxymethylnitrosamino)-1-(3-pyridyl)-1\-butanone (HO-methyl NNK). The reactive intermediate spontaneously yields 4-(3-pyridyl)-4-oxobutyl diazohydroxide, which can pyridyloxobutylate DNA (Hecht, 1998; Jalas et al., 2005).

Some of the metabolites of NNK are further metabolized in a reaction catalyzed by UDP-glucuronosyltransferases (UGT). At present, 4 NNK-related glucuronides have been reported. These are HO-methyl NNK glucuronide, 4-hydroxy-1-(3-pyridyl)-1-butane (HPB) glucuronide, and O- and N-glucuronides of NNAL. HPB is a breakdown product of HO-methyl NNK and NNAL is a carbonyl reduction product of NNK. Glucuronidation is generally considered to be a detoxification reaction so that the glucuronide of NNK-related metabolites is thought to be inactive (Morse et al., 1990; Hecht et al., 1993; Murphy et al., 1995; Wiener et al., 2004; Chen et al., 2008). However, it is of interest to note that the glucuronide of HO-methyl NNK, a stable form of a reactive intermediate, can travel to target organs for NNK-induced carcinogenesis. The glucuronide is then subjected to β-glucuronidase cleavage that could reproduce a reactive intermediate. Regeneration of HO-methyl NNK could play an important role in the induction of target organ-specific carcinogenicity (Murphy et al., 1995, 1997).

Very recently, our laboratory investigated the tissue distributions of HO-methyl NNK glucuronide and revealed that the glucuronide was detected in the target organs of NNK carcinogenesis (Nishiyama et al., 2014). We also reported that UGT 2 subfamily enzymes are involved in the glucuronidation of HO-methyl NNK using Gunn rats, which hereditarily lack the expression of UGT 1A subfamily enzymes. However, we did not have any information about the glucuronide, that is, whether the glucuronide serves as the transport form of the reactive intermediate of NNK to the target organs (Fig. 1). In the present study, we employed A/J mice and C57BL/6J mice, strains which are well known to have different susceptibilities to NNK-induced carcinogenesis, and investigated differences in the metabolism and stability of HO-NNK glucuronide in the tissues of the two strains.

**MATERIALS AND METHODS**

**Materials**

Male A/J and C57BL/6J mice (6 weeks old) were purchased from Japan SLC (Shizuoka, Japan). Oasis HLB cartridges (1 ml, 30 mg) were purchased from Waters (Milford, MA, USA). Acetonitrile and ammonium acetate were purchased from Wako (Tokyo, Japan). 4-Trifluoromethylumbelliferyl glucuronide potassium salt

![Fig. 1](image-url)  
**Fig. 1.** Proposed metabolic pathway of NNK focusing on the formation of HO-methyl NNK and HO-methyl NNK glucuronide. Structures in brackets are hypothetical intermediates. CYP, cytochrome P450; HPB, 4-hydroxy-1-(3-pyridyl)-1-butane; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butane; HO-methyl NNK, 4-hydroxymethyl NNK; UGT, UDP-glucuronosyltransferase.
(HFC-glucuronide) was purchased from Sigma-Aldrich (St. Louis, MO, USA). 4-Trifluoromethylumbelliferone (HFC) was purchased from Acros Organics (Geel, Belgium). Phenobarbital (PB) and pentobarbital were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). HPB and NNK were purchased from Toronto Research Chemicals, Inc. (North York, Ontario, Canada). All of the other reagents were of the highest grade commercially available.

Animal treatment and sample collection

Animal protocols were approved by the Institutional Care and Use Committee of Tokyo University of Pharmacy and Life Sciences. Male A/J and C57BL/6J mice (20-25 g) were provided with food and water ad libitum and were maintained under controlled temperatures and lighting. Mice were divided into PB-induced and control groups of 3 each. The PB-induced group was administered PB (10 mg/kg) intraperitoneally every 24 hr for 3 days. Twenty-four hours after the last dose, NNK (50 mg/kg) was administered intraperitoneally to both PB-induced and control groups. Kidney, liver and lung tissues were obtained from mice that were sacrificed by an overdose of pentobarbital 15, 30, 60 and 120 min after administration. Tissues were pooled from 3 mice in each group. For urine collection, the mice were placed in metabolic cages in groups of 5, and pooled 24-hr urine collections were made. Urine was collected into 50 ml conical tubes, cooled by ice-cold water, filtered through a membrane filter and subjected to LC/MS analysis.

Sample preparation

Pooled minced tissues were each homogenized in approximately 3 volumes of ice-cold saline. The homogenates were centrifuged at 12,000 × g for 15 min at 4°C, and 1 ml of the resulting supernatant fluids was transferred into another centrifuge tube. 100 μl of 10% trichloroacetic acid solution (TCA) was added to the fluid and centrifuged at 12,000 × g for 15 min, and the resulting supernatants were subjected to solid-phase extraction.

Solid-phase extraction and HPLC-ESI-Triple Quadrupole-MS (LC/MS/MS) analysis

Solid-phase extraction and subsequent HPLC-ESI-Triple Quadrupole-MS (LC/MS/MS) analysis were performed as described previously (Nishiyama et al., 2014).

Quantification of HO-methyl NNK glucuronide

LC/MS/MS quantification of HO-methyl NNK glucuronide was performed as described previously (Nishiyama et al., 2014).

Purification of HO-methyl NNK glucuronide

HO-Methyl NNK glucuronide in urine was isolated and purified by a series of HPLC separations as follows. The urine was loaded onto a Superiex ODS column (10 x 250 mm, 5 μm; Shiseido, Tokyo, Japan) and eluted at a flow rate of 2.5 ml/min with a 5 to 25% (v/v) linear gradient of acetonitrile (1%/min) in 100 mM ammonium acetate (pH 5.0). HO-Methyl NNK glucuronide was eluted at a retention time of 16 min from the column used for the first step of HPLC. For further purification, the eluate containing HO-methyl NNK glucuronide was subjected to the second step of HPLC on a Docosil 300 column (4.6 x 150 mm, 5 μm; Senshu Scientific Co., Tokyo, Japan) eluted at a flow rate of 0.5 ml/min with a 5 to 20% (v/v) linear gradient of acetonitrile (1%/min) in 100 mM ammonium acetate (pH 5.0). The third step of HPLC on a C6H5-1152-N column (4.6 x 150 mm, 5 μm; Shiseido) eluted at a flow rate of 0.5 ml/min with a 5 to 15% (v/v) linear gradient of acetonitrile (0.5%/min) in 100 mM ammonium acetate (pH 5.0). HO-Methyl NNK glucuronide was purified by the fourth step of HPLC. The eluate was subjected to the fourth step of HPLC for purification on the Mightysil NH2 column (4.6 x 150 mm, 5 μm; Kanto Chemical Co., Tokyo, Japan) eluted at a flow rate of 0.5 ml/min with acetonitrile (0.5%/min) in 100 mM ammonium acetate for a 80:20 ratio of acetonitrile to 100 mM ammonium acetate (pH 5.0). Fractions were collected every minute, and the elution of HO-methyl NNK glucuronide was monitored by LC/MS/MS.

Stability of HO-methyl NNK glucuronide

To assess the stability of HO-methyl NNK glucuronide and HFC-glucuronide, the glucuronides (final, 200 μM each) were added to 100 mM phosphate buffer at pH 6.2, 7.4 and 8.0, each containing liver homogenate (5 mg/ml). Incubation was performed at 37°C for 0 hr, 2 hr, 4 hr, 6 hr and 24 hr, and the reaction was terminated by the addition of 10% TCA. After centrifugation at 12,000 x g for 10 min, the supernatant was subjected to LC/MS/MS analysis.

RESULTS AND DISCUSSION

HO-Methyl NNK glucuronide is a unique glucuronide compared to the other glucuronides because the glucuronic acid is linked to the hydroxyl group of the reactive intermediate metabolically formed from NNK so that the formation of HO-methyl NNK glucuronide can allow stabilization of HO-methyl NNK (Murphy et al., 1995, 1997; Nishiyama et al., 2014) (Fig. 1). It is possible that HO-methyl NNK glucuronide is transported to the tar-
get organs of NNK carcinogenesis, and then subjected to hydrolysis causing the release of the reactive intermediate. This is one of the possibilities for the organ-specific carcinogenicity of NNK, which is dependent on the transport of HO-methyl NNK glucuronide and hydrolysis of the glucuronide in the target organs (Murphy et al., 1997; Nishiyama et al., 2014). Generally, UGT-dependent glucuronidation is thought to be an important detoxification process. The formation of HO-methyl NNK glucuronide gives a tantalizing insight into whether the glucuronide is the detoxification product or the transporter of the reactive intermediate. In this study, to investigate whether HO-methyl NNK glucuronide is a detoxified metabolite or acts as the transporter of the reactive intermediate in NNK carcinogenesis, we compared differences in the metabolism of HO-methyl NNK glucuronide between A/J mice and C57BL/6J mice because susceptibility to NNK tumorigenesis differs between the strains (Devereux et al., 1993; Kawano et al., 1996).

HO-Methyl NNK glucuronide concentrations in urine and tissues were determined in the PB-induced and control mice after injection of NNK intraperitoneally (50 mg/kg) (Figs. 2 and 3). In the A/J mice, which are highly susceptible to NNK-induced carcinogenesis, a small amount of HO-methyl NNK glucuronide was detected in urine from the control group. In contrast, in the C57BL/6J mice in the control group, which are resistant to tumorigenesis induced by NNK, the amount of HO-methyl NNK glucuronide was detected to be more than 20 times higher than in the control A/J mice urine. In the PB-induced A/J mice, urinary excretion of HO-methyl NNK glucuronide was increased and reached the same level as in the C57BL/6J control mice. PB-pretreatment also increased urinary excretion of HO-methyl NNK glucuronide in C57BL/6J mice. Our previous results using Wistar rats showed that urinary excretion of HO-methyl NNK was only increased in rats after induction of enzymes for drug metabolism. Interestingly, in the case of C57BL/6J mice, HO-methyl NNK glucuronide was detected not only in the PB-induced mice but a considerable amount of HO-methyl NNK was detected in the control C57BL/6J mice.

Figure 3 shows the distribution of HO-methyl NNK glucuronide in tissues such as kidney, liver and lung at 15, 30, 60 and 120 min post-administration. In the control C57BL/6J mice, HO-methyl NNK glucuronide was detected in the kidney, lung and liver. In the control A/J mice, HO-methyl NNK glucuronide was detected in the kidney and liver, but not in the lung. In both strains, within 15 min after administration of NNK, kidney and liver HO-methyl NNK glucuronide concentrations had reached the highest point. The concentration of HO-methyl NNK glucuronide in the kidney of C57BL/6J mice was the highest among the tissues investigated. In both PB-induced strains, HO-methyl NNK glucuronide was also detected in kidney and liver. It is interesting that the glucuronide was still not detected in the lung of A/J mice after PB-induction. In both strains, the concentrations of HO-methyl NNK glucuronide in the tissues decreased quickly after reaching Cmax.

In order to evaluate the stability of HO-methyl NNK in tissues, we investigated the degradation of HO-methyl NNK glucuronide using different pH conditions and liver homogenates (Fig. 4). In both strains, more than 60% of the HO-methyl NNK glucuronide remained in all tissue homogenate preparations at pH 7.4 after 2 hr of incubation, and at 6 hr of incubation more than 40% remained. At pH 6.2, HO-methyl NNK glucuronide was less stable than at pH 7.4 and rapidly degraded. We also investigated the stability of HFC-glucuronide as a control substance. Under the same incubation conditions, HFC-glucuronide was less stable than at pH 7.4 and rapidly degraded. These results indicated that HO-methyl NNK glucuronide is relatively stable in tissues under physiological condi-

![Fig. 2. Cumulative urinary excretion of HO-methyl NNK glucuronide. Urine samples were collected for 24 hr after intraperitoneal administration of NNK (50 mg/kg) to PB-induced A/J and C57BL/6J mice and control A/J and C57BL/6J mice. Data collection and analysis were described in Materials and Methods.](image-url)
tions so that the formation of the glucuronide plays an important role in the detoxification of HO-methyl NNK, not as a carrier of the reactive intermediate. Taken together, HO-methyl NNK glucuronide is likely to be a detoxified metabolite.

The present study showed that the ability to form HO-methyl NNK glucuronide in C57BL/6J mice was superior to that in A/J mice. Our results could explain the reason for the differences in susceptibility to NNK tumorigenesis between the two strains, because once HO-methyl NNK is formed in tissues, C57BL/6J mice have a high ability to form HO-methyl NNK glucuronide so that the reactive intermediate is readily excreted into urine in a stable form. In contrast, A/J mice have a lower ability to form HO-methyl NNK glucuronide, which might not prevent pyridyoxobutylation to DNA. Indeed, HO-methyl NNK glucuronide was not detected in the A/J mouse lung, which is a major target organ of NNK tumorigenesis.

Our previous study showed that UGT2B isoforms mainly contributed to the glucuronidation of HO-methyl NNK.
NNK in Wistar rats (Nishiyama et al., 2014). Buckley and Klaassen (2006) reported tissue expression patterns of Ugt mRNA in C57BL/6J mice. The report revealed that Ugt2b isoforms such as Ugt2b1, 2b5, 2b34, 2b35, 2b36, 2b37 and 2b38 were constitutively expressed in the liver and kidney. In the lung, Ugt2b35 and 2b36 were expressed constitutively. In addition, some of these Ugt2b isoforms, namely Ugt2b1 and 2b36, were shown to be

![Graphs showing effect of pH on hydrolysis of HO-methyl NNK glucuronide and HFC-glucuronide in liver homogenates.](image_url)

**Fig. 4.** Effect of pH on hydrolysis of HO-methyl NNK glucuronide (A, B) and HFC-glucuronide (C, D) in liver homogenates. HO-Methyl NNK glucuronide or HFC-glucuronide was incubated in liver homogenates under different pH conditions. After indicated incubation time, the reaction was stopped and HPB or HFC formation was analyzed as described in Materials and Methods.
PB-inducible in the liver (Buckley and Klaassen, 2009). All of the available information suggests that Ugt2b1 and 2b36 are candidates as enzymes that catalyze HO-methyl NNK glucuronidation. However, we still cannot determine what is the most important factor for the production of the HO-methyl NNK glucuronide, namely, activity for the hydroxylation of the α-methyl group of NNK by CYPs or glucuronidation of HO-methyl NNK by UGTs or both enzymes.

Our results also provided evidence that HO-methyl NNK glucuronide was easily degraded under acidic conditions. It is of interest to note the relation between bladder tumorigenesis and the stability of HO-methyl NNK glucuronide in acidic urine. Our laboratory is currently investigating the stability of HO-methyl NNK glucuronide in bladder tissue.

REFERENCES


