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Dihydromethysticin (DHM) blocks tobacco carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)-induced O6-methylguanine independent of aryl hydrocarbon receptor (AhR) in C57BL/6 female mice

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Dihydromethysticin (DHM) blocks tobacco carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)-induced O^6-methylguanine independent of aryl hydrocarbon receptor (AhR) pathway in C57BL/6 female mice

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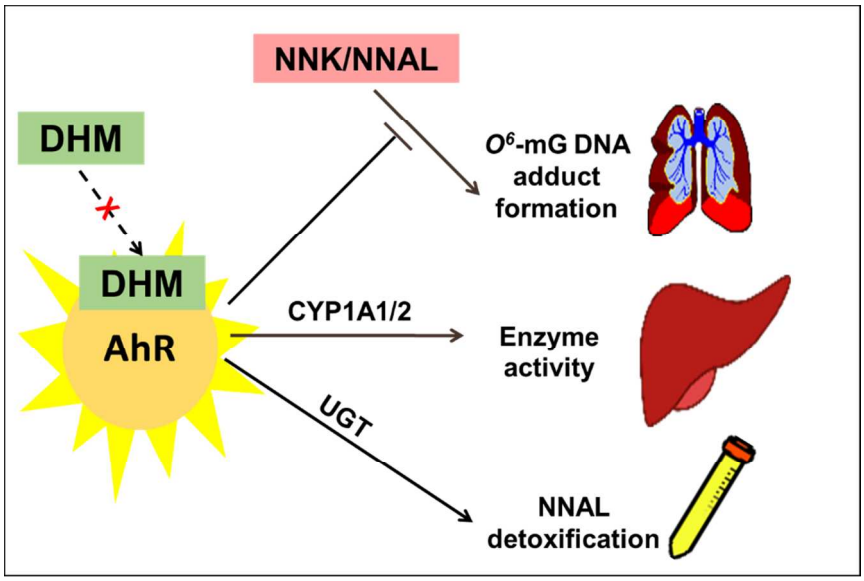

ABSTRACT

4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) is a key carcinogen responsible for tobacco smoke-induced lung carcinogenesis. Among DNA damages caused by NNK and its metabolite 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), O^6-methylguanine (O^6-mG) is likely the most carcinogenic in A/J mice. Results of our previous studies showed that O^6-mG and other NNAL-derived DNA damages were preferentially reduced in the lung of female A/J mice upon the dietary treatment with dihydromethysticin (DHM), a promising lung cancer chemopreventive agent from kava. Such a differential blockage may be mediated via increased NNAL glucuronidation and thereof leading to its detoxification. The potential of the aryl hydrocarbon receptor (AhR) as an up-stream target of DHM mediating these events was evaluated herein using $Ahr^{+/-}$ and $Ahr^{-/-}$ C57BL/6 female mice because DHM was reported as an AhR agonist. DHM (0.05, 0.2 and 1.0 mg/g of diet) and dihydrokavain (DHK, an inactive analog, 1.0 mg/g of diet) were given to mice for seven days, followed by a single i.p. dose of NNK at 100 mg/kg of bodyweight. The effects of DHM on the amount of O^6-mG in the lung, on the urinary ratio of glucuronidated NNAL (NNAL-Gluc) and free NNAL, and on CYP1A1/2 activity in the liver microsomes were analyzed. As observed in A/J mice, DHM treatment significantly and dose-dependently reduced O^6-mG in the target lung tissue, but there were no significant differences in O^6-mG reduction between mice from $Ahr^{+/-}$ and $Ahr^{-/-}$ backgrounds. Similarly, in both strains, DHM at 1 mg/g of diet significantly increased the urinary ratio of NNAL-Gluc to free NNAL and CYP1A1/2 enzymatic activity in liver with no changes detected at lower DHM dosages. Since none of these effects of DHM were dependent of *Ahr* status, AhR clearly is not the up-stream target for DHM.

INTRODUCTION

As the leading cause of cancer-related death, lung cancer accounted for about 160,000 deaths in the United States and more than 1.6 million deaths worldwide annually.[1] Due to the lack of robust early diagnosis and effective treatment, the five-year survival rate of lung cancer patients has been around 15 – 18% for decades.[2] Although developing effective therapy and early diagnosis is important in the clinical management of lung cancer, it is also essential to develop chemopreventive agents against this disease.

Tobacco use accounts for 85 – 90% of lung cancer incidence.[3] Many chemicals detected in tobacco smoke have been classified as human carcinogens.[4] Among these, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK, a tobacco-specific nitrosamine) is a well-studied pulmonary carcinogen.[5] Upon cytochrome P450 (CYP450) enzyme-mediated hydroxylation, NNK generates reactive intermediates that can form DNA damages. Alternatively, NNK can be metabolically reduced to 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), which will be bioactivated by CYP450 to form DNA damages (Scheme 1). These DNA damages, if not repaired, can lead to gene mutations and initiate lung tumorigenesis.[6] Therefore, chemical entities that can block NNK/NNAL-induced DNA damages have the potential to prevent lung carcinogenesis.

Our earlier studies showed that dihydromethysticin (DHM), a natural product from *Piper methysticum* (kava), could block NNK-induced lung tumorigenesis in A/J mice; provided it was given during the tumor initiation phase.[7,8] DHM at a dose of 0.05 mg/g in diet was able to reduce lung adenoma multiplicity by 97%, with a significant reduction in DNA adduct O^6-mG (likely the most carcinogenic DNA damage in A/J mice[9]). Further studies suggested that the reduction in O^6-mG by DHM may originate from the increased detoxification of NNAL.[10]

Specifically, dietary DHM increased the relative abundance of 4-(methylnitrosamino)-1-(3-pyridyl)-1-(*O*-*β*-D-glucopyranuronosyl)butane (NNAL-*O*-Gluc) in A/J mouse urine, which is likely mediated via the enhanced NNAL glucuronidating activity.[10]

NNAL glucuronidation is catalyzed by UDP-glucuronosyltransferases (UGTs).[11] UGTs are typically regulated by several transcriptional factors, including pregnane X receptor (PXR), constitutive androstane receptor (CAR)[12,13] and aryl hydrocarbon receptor (AhR).[14] Recently Li *et al.* reported that DHM and methysticin, another structurally similar kavalactone in kava that also blocks NNK/NNAL-induced O^6-mG formation in A/J mice,[8] could activate the AhR pathway *in vitro.*[15] AhR is a ligand-dependent transcriptional factor highly expressed in the lung.[16] Upon the binding of its agonist, AhR translocates to the nucleus, dimerizes with ARNT, followed by the binding to the xenobiotic-response element (XRE) of drug-metabolizing genes to activate their transcription.[17] Besides UGTs and other phase II enzymes, phase I enzymes, particularly CYP1A1, are also dominantly regulated by AhR. While being essential for normal metabolism and other regular cellular functions,[18] CYP1A1/2 enzymes may also be involved in the bioactivation of certain carcinogens, such as benzo[*a*]pyrene (BaP)[19] and 2-amino-1-methyl-6-phenylimidazo(4,5-b)pyridine (PhIP).[20] Due to these complicated roles, the induction of CYP1A1/2 has been carefully evaluated in many herb-drug or drug-drug interactions.[21] Indeed, Li *et al.* demonstrated that DHM and methysticin could induce CYP1A1 at mRNA, protein and function levels in Hepa1c1c7 cells in an AhR-dependent manner.[15] Several *in vivo* studies also reported that high dosage of kava extract led to increased mRNA and protein of hepatic CYP1A1 in rodents.[22,23] These results overall suggest that AhR might be one possible up-stream target of DHM, which may activate UGTs and result in enhanced detoxification of NNAL that would account for its effect against NNK/NNAL-induced DNA damage in A/J mice. At the same time,

DHM may enhance the metabolic activation of other carcinogens and increase the risk of herb-drug interaction upon activating AhR and inducing CYP1A1/2.[24]

Given DHM's outstanding efficacy in blocking NNK/NNAL-induced DNA damage and lung tumorigenesis, there is an urgent need to elucidate its mechanism of action. In this study, we investigated the role of AhR as a potential target of DHM using *Ahr*$^{+/-}$ and *Ahr*$^{-/-}$ C57BL/6 mice. We analyzed whether AhR deficiency has any effect on 1) DHM-mediated reduction in O^6-mG in target lung tissues, 2) increased NNAL-*O*-glucuronidation in urine, and 3) induction of CYP1A1/2 in the liver. Our results revealed that AhR status has no influence on any of these DHM-mediated effects, demonstrating that AhR is not the upstream target.

REAGENTS AND METHODS

Caution: NNK and NNAL are human carcinogens. Personnel handlings are expected to have appropriate safety measures.

Chemicals and reagents NNK, [$^{13}C_6$]NNK, [CD_3]O^6-mG and [4-CD_2,CD_3]NNAL-*O*-Gluc were purchased from Toronto Research Chemicals (Toronto, ON, Canada). NNAL and [$^{13}C_6$]NNAL were synthesized from NNK or [$^{13}C_6$]NNK via sodium borohydride reduction.[10] (±)-DHM and (±)-DHK were synthesized in-house with a slight modification of a reported procedure.[25] AIN-G powdered diet was purchased from Harlan Teklad (Madison, WI). Recombinant β-glucuronidase was purchased from Sigma-Aldrich (St. Louis, MO). Ethoxyresorufin was purchased from Sigma-Aldrich. NADPH was purchased from RPI (Mount Prospect, IL). The qPCR primers were purchased from IDT (Coralville, IA) and the sequences are as follows, *Gapdh* sense 5'-AACTTTGGCATTGTGGAAGG-3', antisense 5'-ACACATTGGGGGTAGGAACA-3'; *Ahr* sense 5'-AGCCGGTGCAGAAAACAGTAA-3', antisense 5'-AGGCGGTCTAACTCTGTGTTC-3'; *Cyp1a1* sense 5'-GGTTAACCATGACCGGGAACT-3',

antisense 5'-TGCCCAAACCAAAGAGAGTGA-3'; *Cyp1a2* sense 5'-TGGAGCTGGCTTTGACACAG-3', and antisense 5'-CGTTAGGCCATGTCACAAGTAGC-3'. All other chemicals or solvents were purchased from either Fisher Scientific (Fairlawn, NJ) or Sigma-Aldrich (St. Louis, MO), unless stated otherwise.

Diet preparation and characterization (±)-DHM and (±)-DHK supplemented AIN-G powdered diets were prepared as per our previously reported procedures.[8] Briefly, (±)-DHM or (±)-DHK was reconstituted in absolute ethanol (50 mL) and then mixed with the AIN-93 G powdered diet (150g). Absolute ethanol (50 mL) was mixed with the AIN-93 G powdered diet (150g) for the control diet. The reconstituted diets were dried under vacuum to remove ethanol and then ground into fine powders. All diets were then mixed well with additional AIN-93 G powdered diet to the desired dose. The abundance of DHM and DHK in the diet was analyzed in triplicate by HPLC and confirmed to be within ±10% of the specified dose.

Animal Studies The animal studies performed herein were approved by the University of Minnesota Institutional Animal Care and Use Committee and conducted following the National Institutes of Health guidelines. Five- to six-week old C57BL/6 $Ahr^{+/-}$ and $Ahr^{-/-}$ female mice were procured from McGill University, Montreal, Canada and housed in the core animal facilities of the Research Animal Resources, University of Minnesota.[26] $Ahr^{+/-}$ mice are phenotypically indistinguishable from wild-type ($Ahr^{+/+}$) mice and are often used as controls when examining the physiological, pathophysiological and toxicological parameters of the AhR rendering $AhR^{+/-}$ mice as suitable controls for this study.[27–29] After one week of acclimation, 18 $Ahr^{+/-}$ and 18 $Ahr^{-/-}$ mice were randomized into six groups each (n=3). From Day 1 to Day 7, they were fed with the corresponding diet. Bodyweight of the mice was measured every two or three days and their food intake was monitored twice a week. On Day 8, except for mice in the

negative control groups, all other mice received a single dose of NNK in saline (100 μL) at 100 mg/kg of body weight via ip injection. Mice in the negative control groups were given saline. Based on the results from our previous studies, four hours after NNK exposure, mice were euthanized with CO_2 overdosing.[10] Mouse sera, urine, lung and liver tissues were collected and stored at -80 °C following our established procedures.[10] For lung and liver tissues, small portions were stored in RNA stabilization solution.

Quantification of *O^6-mG* DNA adduct in the lung tissues DNA isolation from the lung tissues and LC-MS/MS quantification were performed following the standard procedure.[7] Briefly DNA was isolated from half of the whole lung tissue of each individual mouse, following Puregene DNA isolation protocol (Qiagen Corp). *O^6*-mG was quantified by liquid chromatography-electrospray ionization/tandem mass spectrometry (LC-ESI-MS/MS) with [CD_3]*O^6*-mG as the internal standard.

Urinary NNAL-*O*-Gluc and free NNAL Quantification Urinary NNAL-*O*-Gluc and free NNAL were quantified via an established LC-MS/MS method.[10] Briefly urine samples were diluted 10^5 times with saline. The diluted samples (0.1 mL each) were mixed with [4-CD_2, CD_3]NNAL-*O*-Gluc and [$^{13}C_6$]NNAL at a final concentration of 5 and 10 ng/mL respectively. LC-MS/MS analysis was performed using an Agilent 1100 series capillary high-pressure liquid chromatography system (Agilent Technologies, Palo Alto, CA) interfaced to a TSQ Quantum Discovery Max triple quadrupole mass spectrometer (Thermo Electron, San Jose, CA). NNAL and NNAL-*O*-Gluc were analyzed simultaneously on a Phenomenex Luna C18 (150 × 0.5 mm, 3 microns) capillary column, under conditions reported before.[10]

Mouse Liver Microsome Preparation and CYP1A1/2 Enzymatic Assay (EROD) Mouse liver microsomes were prepared following the standard protocol.[10] Briefly, liver tissue (~250 mg)

was manually homogenized in ice cold microsome buffer (1 mL, 50 mM Tris·HCl, 1.15% KCl, 1 mM EDTA, pH 7.0). The resulting homogenate was centrifuged at 10,000 RPM at 4°C for 30 minutesusing 70.1 Ti rotor (Beckman Coulter, Brea, CA). The supernatant was collected and further centrifuged at 40,000 RPM at 4°C for 90 minutes. The pellet was resuspended in ice cold microsome buffer (100 μL). Upon protein quantification by BCA method, microsome was diluted to a concentration of 5 mg/mL total protein, aliquoted and stored at -80°C until use.

Aliquoted microsomes were thawed on ice right before the enzymatic assay. 7-Ethoxy-resorufin-*O*-deethylation (EROD) enzymatic assays were conducted following the standard protocols in 96-well plates.[30] Briefly, microsome was incubated with 7-ethoxy-resorufin under 37°C for 10 min followed by the addition of NADPH. With a final reaction volume of 100 uL, each well contained 0.2 mg/mL microsomal protein, 1 mM NADPH, and 1 μM 7-ethoxyresorufin in microsome buffer. Fluorescence intensity in each well was continuously monitored by a Tecan microplate reader (GENios Pro) with an excitation wavelength of 535 nm and an emission wavelength of 590 nm (20 measurements each well in 15min). During this period of time, the increase in fluorescence intensity was linear, suggesting minimal changes of the enzymatic activity and substrate concentration (data not shown). Standard curve used for unit conversion was generated with resorufin purchased from Santa Cruz Biotechnology (Dallas, TX).

Western Blotting Analyses Liver (~50mg) and lung (~10mg) tissue samples were first homogenized using TissueRuptor from Qiagen with T-PER Tissue Protein Extraction Reagent from Thermo Fisher, supplemented with protease inhibitors (Thermo Fisher) and phosphatase inhibitors cocktail (Cell Signaling, Danvers, MA). Protein concentration was determined by BCA method. Western blotting was conducted with 50 μg liver tissue lysates or 12.5 μg lung

tissue lysates per well. Primary antibody for AhR was purchased from Enzo Life Sciences (Farmingdale, NY) (cat. BML-SA210), 1:5000. HRP-linked β-actin antibody was obtained from Sigma-Aldrich (cat. A3854), 1:25000. HRP-linked anti-rabbit IgG from Cell Signaling was used as secondary antibody for AhR with a dilution factor of 1:3000. β-actin served as loading control and protein bands were visualized by the chemiluniscence method.

Quantitative Reverse Transcription PCR Liver and lung tissue samples were saved in RNA stabilization solution. Total RNA was isolated using RNeasy Mini kit (Qiagen) following manufacturer's protocol. cDNA was synthesized from 2 µg total RNA each using SuperScript Vilo cDNA Synthesis kit (Invitrogen, Carlsbad, CA). qPCR was done on a StepOnePlus™ Real-Time PCR System using SYBR Green PCR Master Mix (Invitrogen, Carlsbad, CA) with the following thermal cycling: 95°C for 5 minutes, proceeded by 45 cycles of 95°C for 15 seconds, and 60°C for 1 minute. *Gapdh* served as a reference gene.

Statistical Analyses Data shown here represent 3 biological repeats (3 mice) with their mean ± standard deviation. Statistical significance is denoted by asterisks with the following definition: not significant, $p\text{-value} > 0.05$; *, $p\text{-value} \leq 0.05$; **, $p\text{-value} \leq 0.01$; ***, $p\text{-value} \leq 0.001$; ****, $p\text{-value} \leq 0.0001$. If not specified with any asterisks, there was no significant difference between compared groups. Except for the results of qRT-PCR, differences between treated and control groups were analyzed by one-way ANOVA, with Dunnett's method used for multiple comparison, at 95% confidence interval. For qRT-PCR results, mRNA expressions between $Ahr^{+/-}$ and $Ahr^{-/-}$ groups were compared with two-tailed student's *t*-test, at 95% confidence interval.

RESULTS AND DISCUSSION

Characterization of *Ahr* Status and Health Monitoring Before randomization and after euthanasia, all mice were genotyped for *Ahr,* following an established procedure (data not shown).[26] In addition, *Ahr* mRNA and protein were analyzed via qRT-PCR and Western blotting respectively in the lung and liver tissues from representative animals (Figure S1). These results validated the *Ahr* status of mice used herein. During the study, there were no significant differences in bodyweight changes or food intake among mice with different treatments (data not shown).

The Effect of *Ahr* Status on Reduction in O^6-mG by DHM in Mouse Lung Tissues O^6-mG is likely the most carcinogenic DNA damage relative to other NNK/NNAL-induced DNA damages in A/J mice[9] and it was effectively reduced by DHM in the target lung tissue.[8] Therefore, pulmonary O^6-mG was analyzed as the representative DNA damage in the $Ahr^{+/-}$ and $Ahr^{-/-}$ mice. As shown in Figure 1, NNK treatment resulted in similar levels of O^6-*mG* adduct formation irrespective of the *Ahr* status and DHM dose-dependently reduced O^6-mG levels in both $Ahr^{+/-}$ and $Ahr^{-/-}$ mice. As expected, the extent of reduction in O^6-mG by DHK was much less, if any, relative to DHM in both genotypes. These results unambiguously demonstrated that the reduction in O^6-mG by DHM is AhR independent.

The Effect of *Ahr* Status on DHM-induced NNAL Glucuronidation Our earlier work revealed that dietary DHM increased the abundance of urinary NNAL-*O*-Gluc relative to free NNAL in A/J mice and enhanced the NNAL glucuorinidating activity in the lung and liver tissues.[10] Mechanistically, increase in UGT-mediated NNAL glucuronidation could lead to enhanced NNAL detoxification and thereof may contribute to the reduction in O^6-mG.[10,31] Since AhR has been reported to transcriptionally regulate UGTs,[14,32] the impact of *Ahr* status on NNAL glucuronidation was evaluated herein. The urinary ratio of NNAL-Gluc and free NNAL

has been used as a convenient parameter to evaluate glucuronidation-mediated NNAL detoxification.[33] Higher urinary ratio of NNAL-Gluc to free NNAL would indicate better detoxification and such a ratio has been used to examine NNK detoxifying capability among smokers.[34] Although both NNAL-*N*-Gluc and NNAL-*O*-Gluc have been detected in human smokers, only NNAL-*O*-Gluc has been detected in A/J mice.[10] In this study we therefore directly quantified NNAL-*O*-Gluc and free NNAL in the mouse urine,[10] and the ratio of NNAL-*O*-Gluc to free NNAL was calculated. As shown in Figure 2, DHM at a dose of 1 mg/g of diet significantly increased the urinary ratio of NNAL-*O*-Gluc to NNAL while DHK had no effect, consistent with our results in A/J mice,[10] supporting that DHM may exert its chemopreventive effects through increasing NNAL detoxification. In agreement with the results of O^6-mG reduction, the detoxification of NNAL by DHM also seemed to be independent of the AhR pathway. Although the extent of increase by DHM in $AhR^{+/-}$ mice seemed to be higher than that in $Ahr^{-/-}$ mice, the basal level was slightly higher in $AhR^{+/-}$ mice as well that further investigation is needed. While DHM was able to reduce the formation of O^6-mG at the dose of 0.05 and 0.2 mg/g of diet, such treatments did not increase NNAL-*O*-Gluc/NNAL ratio (data not shown). A simple explanation could be glucuronidation of NNAL is not the major mechanism responsible for the reduction of NNK/NNAL-induced O^6-mG by DHM treatment. Alternatively, the urinary ratio of NNAL-*O*-Gluc/NNAL may not accurately reflect the target lung tissue since liver is the major metabolizing tissue. Our earlier work showed that dietary DHM treatment only preferentially reduced NNK/NNAL-induced DNA damage in the target lung tissue not the liver tissue.[8] It is possible that lower dosages of DHM may be sufficient to enhance NNAL glucuronidation in the target lung tissue while higher dosages of DHM may be needed to enhance NNAL glucuronidation in the liver, which requires further investigation.

The Effect of *Ahr* Status on CYP1A1/2 Activity by DHM in Mouse Liver Microsome In addition to its chemopreventive potential, kava usage has been linked to hepatotoxicity in humans.[35] Here we characterized the impact of dietary DHM on CYP1A1/2 to further explore its safety. We focused on CYP1A1/2 in this study mainly because these two CYP isozymes are dominantly regulated by AhR[36] and Li *et al.* has demonstrated CYP1A1 activation by DHM in an AhR dependent manner *in vitro*.[15] In addition, CYP1A1 has been reported to be up-regulated *in vivo* upon high dosages of kava treatment.[22] Moreover, as discussed before, CYP1A1/2 may activate other carcinogens to potentially promote carcinogenesis.[20,37]

Since DHM was given to mice in diet and its impact on *Cyp1a1* and *Cyp1a2* mRNA was expected to be temporal, we attempted to quantify CYP1A1 and CYP1A2 proteins in the liver tissues via Western blotting. Due to the lack of well-validated isoform specific antibodies for CYP1A1 and CYP1A2, Western blotting results were inconclusive (data not shown). In the next step, enzymatic activity of CYP1A1/2 was evaluated through 7-ethoxy-resorufin-*O*-deethylation (EROD) assay (Figure 3).[30] In spite of previous *in vitro* evidence showing CYP1A1 induction by DHM via the AhR pathway,[15] DHM's effect on the liver microsomal CYP1A1/2 activity (EROD) was independent of *Ahr* status in this study. Such a discrepancy can be due to many reasons, including *in vitro* and *in vivo* models, tissue differences, dosage, metabolism, temporal and spatial distribution of DHM. In comparison to the NNK control groups, DHM at the highest dose (1 mg/g) significantly increased EROD activity; while the extent of increase induced by DHK was much weaker and statistically non-significant. With lower doses of DHM (0.05 mg/g or 0.2 mg/g), liver microsomal CYP1A1/2 activities did not show significant difference when compared to NNK control groups in both $Ahr^{+/-}$ and $Ahr^{-/-}$ mice.

It appeared that the basal level of liver microsomal EROD activity was lower in the *Ahr*$^{-/-}$ mice relative to that in the *Ahr*$^{+/-}$ mice, although the differences were not statistically significant, suggesting that even in the liver, AhR may contribute to the basal levels of CYP1A1/2.

CONCLUSION

Since the effect of DHM on pulmonary NNK/NNAL-induced O^6-mG, urinary ratio of NNAL-*O*-Gluc/NNAL, and liver microsomal EROD were similar in both Ahr$^{+/-}$ and Ahr$^{-/-}$ C57BL/6 mice, AhR clearly is not the up-stream target of DHM. Because of the low sensitivity of C57BL/6 mice to NNK/NNAL-induced lung tumorigenesis in comparison to the A/J mice and the lack of *Ahr*$^{-/-}$ A/J mice, we were unable to evaluate the effect of *Ahr* status on DHM's tumor reduction potential. It is clear that O^6-mG formation is unlikely the only molecular basis responsible for NNK-induced lung tumorigenesis, particularly in C57BL/6 mice.[38] Nevertheless, a strong positive correlation between O^6-mG levels and lung tumor multiplicity in A/J mice has been observed.[9] It is reasonable to speculate that DHM may block NNK/NNAL-induced lung tumorigenesis in A/J mice independent of the AhR pathway, which needs further validaton.

It was also observed that DHM at 0.2 or 0.05 mg/g of diet, while retaining complete chemopreventive effect, did not cause any induction of CYP1A1/2 activity in the liver microsome. DHM therefore has a decent therapeutic window to effectively block lung tumorigenesis without enhancing CYP1A1/2 activity. In addition, flavokawains A and B, not DHM, have been recently identified by us and others as the compounds potentially responsible for kava's hepatotoxic risk.[39] Nevertheless, comprehensive studies are needed to systematically assess the physiological and pathological impacts of DHM on CYP1A1/2 and other drug metabolizing enzymes.

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ABBREVIATIONS LIST

DHM, dihydromethysticin; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; AhR, aryl hydrocarbon receptor; NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; O^6-mG, O^6-methylguanine; DHK, dihydrokavain; NNAL-*O*-Gluc, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol glucuronide; UGT, UDP-glucuronosyltransferase; PXR, pregnane X receptor; CAR, constitutive androstane receptor; XRE, xenobiotic-response element; BaP, benzo[a]pyrene; PhIP, 2-Amino-1-methyl-6-phenylimidazo(4,5-b)pyridine; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; NADPH, reduced form of nicotinamide adenine dinucleotide phosphate; EROD, 7-ethoxy-resorufin-*O*-deethylation; qRT-PCR, quantitative reverse transcription polymerase chain reaction; and ANOVA, analysis of variance.

SUPPORTING INFORMATION

Confirmation of the *Ahr* status of lung and liver tissues from representative mice. This material is available free of charge via the Internet at http://pubs.acs.org

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FIGURE LEGEND

Figure 1. Effect of *Ahr* status on DHM and DHK on NNK/NNAL-induced O^6-mG in the target lung tissues. Data represents mean ± standard deviation of 3 mice per treatment group. Statistical comparison was made with the NNK-only treatment group in each strain of mice via one-way ANOVA.

Figure 2. Effect of *Ahr* status on DHM-induced NNAL glucuronidation in urine. Ratio of NNAL-*O*-Gluc : free NNAL was used as a measurement of NNAL glucuronidation. Both DHM and DHK were at a dose of 1 mg/g diet. Data represents mean ± standard deviation of 3 mice per treatment group. Statistical comparison was made with the NNK-only treatment group in each strain of mice via one-way ANOVA.

Figure 3. Effect of *Ahr* status on CYP1A1/2 activity (EROD) in the liver microsome. Data represents mean ± standard deviation of 3 mice per treatment group. Statistical comparison was made with the NNK-only treatment group in each strain of mice via one-way ANOVA.

FIGURES

Figure 1:

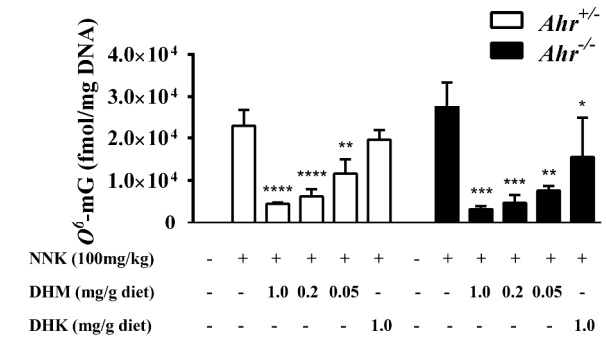


Figure 2

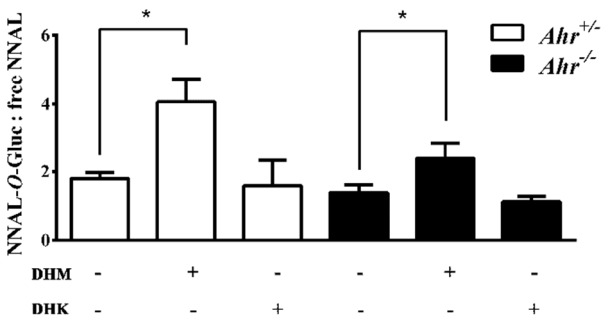
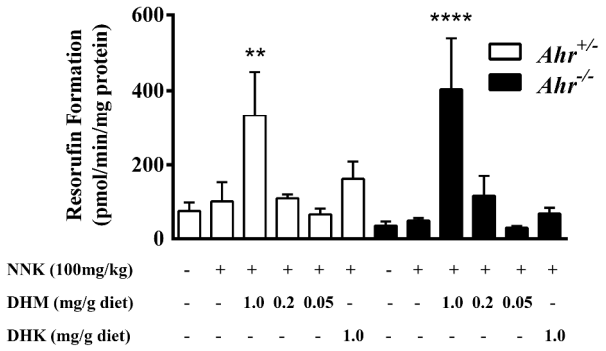


Figure 3


600
400
200
0
Resorufin Formation
(pmol/min/mg protein)
**
****
Ahr+/-
Ahr-/-
NNK (100mg/kg) - + + + + + - + + + + +
DHM (mg/g diet) - - 1.0 0.2 0.05 - - - 1.0 0.2 0.05 -
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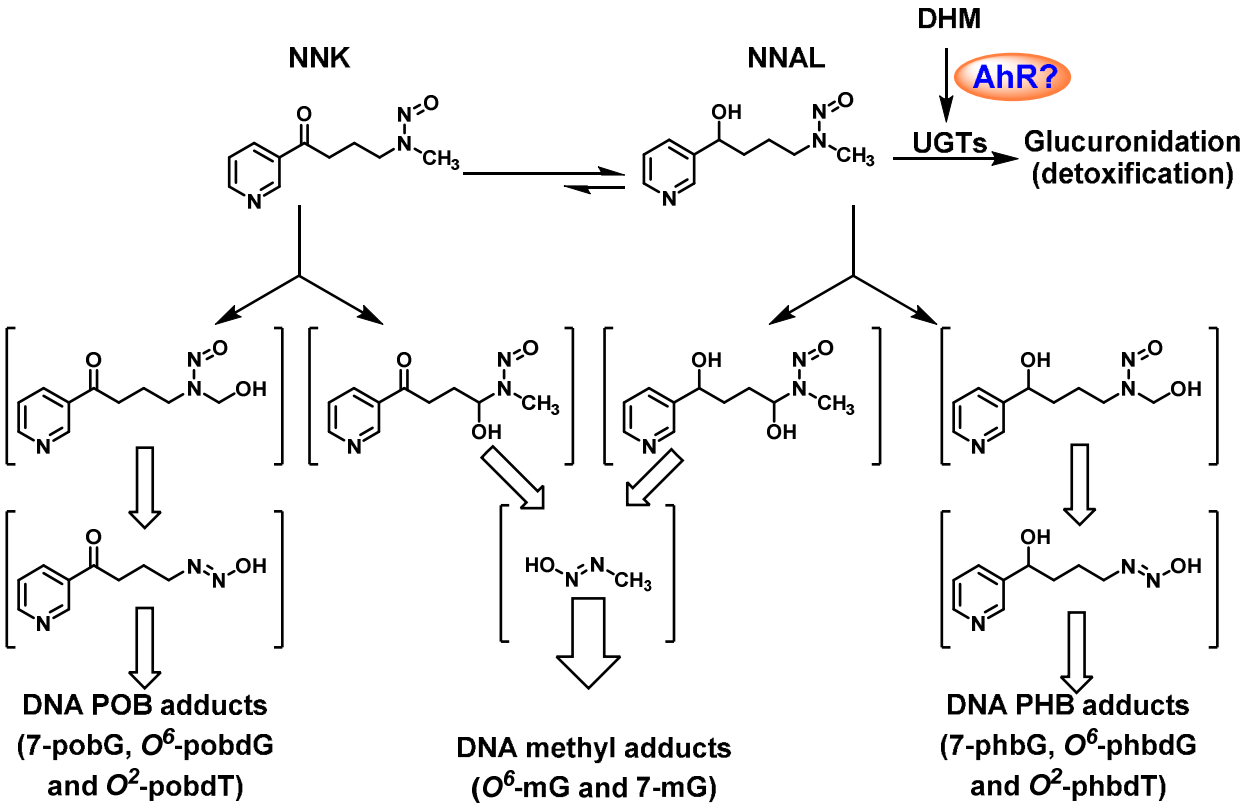
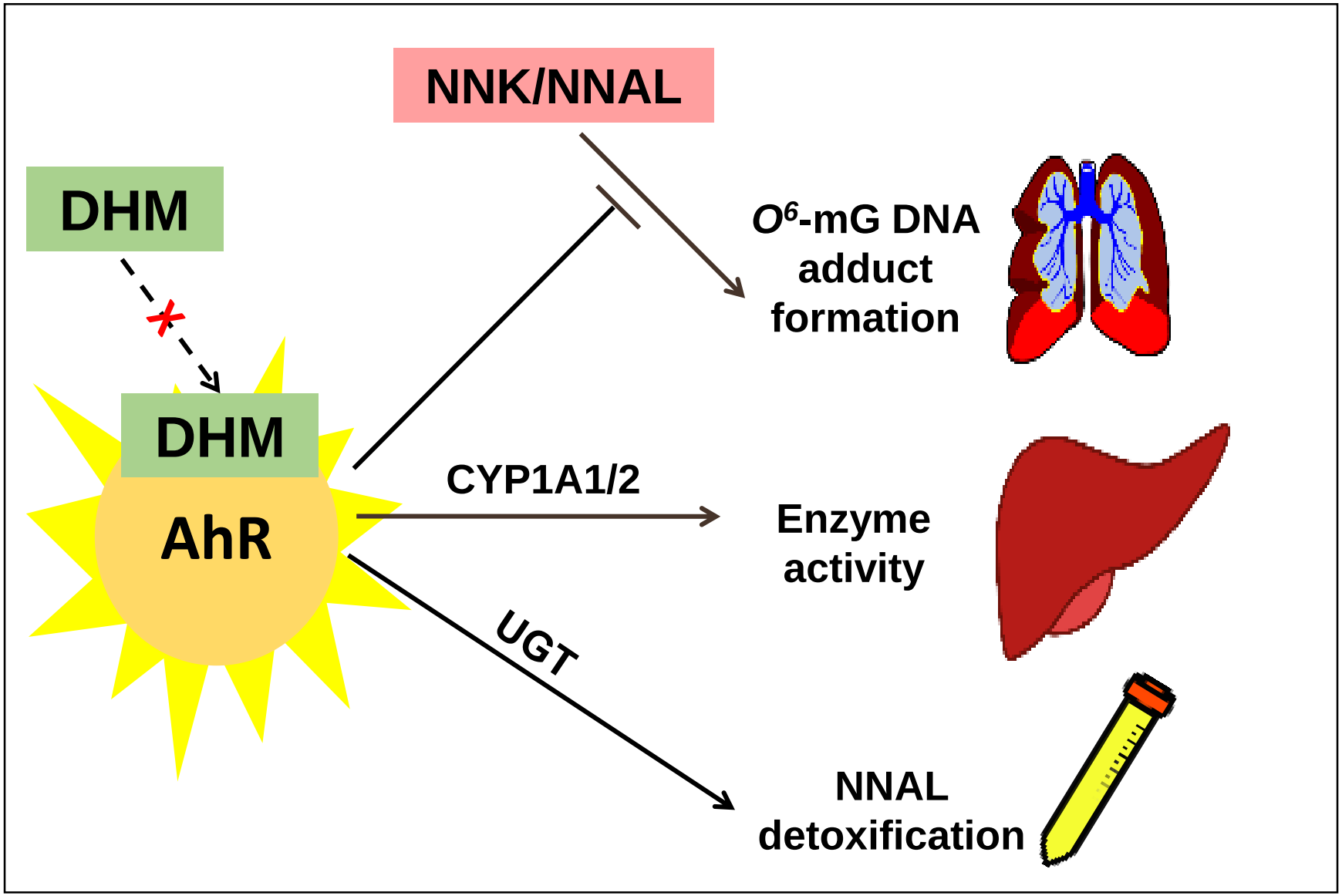


Scheme 1. Putative mechanism of action of DHM via activating the AhR pathway to enhance NNAL glucuronidation, which reduces NNK and NNAL-induced DNA damage and blocks lung carcinogenesis.


NNK/NNAL
DHM
DHM
AhR
O^6-mG DNA adduct formation
CYP1A1/2
Enzyme activity
UGT
NNAL detoxification