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Mutation Research 494 (2001) 21–29

Genetic Toxicology and Environmental Mutagenesis

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# The effect of tobacco smoke, nicotine, and cotinine on the mutagenicity of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL)

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Received 17 May 2000; received in revised form 23 March 2001; accepted 28 March 2001

**Abstract**

4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) is a rodent carcinogen that is metabolically derived from carbonyl reduction of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). NNAL can be pyridine N-oxidized to form NNAL-N-oxide, or conjugated to form NNAL–glucuronide — non-genotoxic metabolites that can be excreted in urine. Alternatively, NNAL can be α-hydroxylated at the methyl and methylene carbons adjacent to the nitroso group to generate electrophiles that can react with biological macromolecules, such as DNA and proteins. Our laboratory has previously demonstrated that the mutagenicity of NNK was significantly inhibited by the aqueous extract of tobacco smoke, as well as pyridine alkaloids in cigarette smoke, such as nicotine, cotinine and nornicotine. Given the structural similarity between NNK and NNAL, and the metabolic activation of both by cytochromes P450, we hypothesized that there may be a similar inhibition of NNAL metabolism, and consequently, inhibition of the mutagenic activity of NNAL by tobacco smoke and its pyridine alkaloid constituents. In the present study, we evaluated the ability of two pyridine alkaloids (nicotine and cotinine) and aqueous cigarette smoke condensate extract (ACTE) to inhibit the mutagenicity of NNAL in *Salmonella typhimurium* strain TA1535 in the presence of a metabolic activation system (S9). Both pyridine alkaloids tested, as well as ACTE, inhibited the mutagenicity of NNAL in a concentration-dependent manner. The observed reductions in mutagenicity were not the result of cell killing due to cytotoxicity. These results demonstrate that tobacco smoke contains pyridine alkaloids, as well as other unidentified constituents that inhibit the mutagenicity of NNAL, a major metabolite of NNK. 

*Keywords:* Tobacco-specific nitrosamines (TSNA); Nicotine; Cotinine; Tobacco smoke; Antimutagenicity; Ames test

## 1. Introduction

Tobacco smoke is a complex chemical mixture containing, among other things, *N*-nitrosamines and pyridine alkaloids, such as nicotine and cotinine. The formation, metabolism, and animal carcinogenicity of the tobacco-related *N*-nitrosamines have been extensively studied [1], and the genotoxicity of nicotine and its major metabolites has been reported [2].

One of the biologically important nitrosamines in tobacco smoke is 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). There are five different metabolic pathways leading to the transformation of NNK. In many tissues, carbonyl reduction of NNK to 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) is the predominant metabolic pathway in vitro and

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1383-5718/01/$ – see front matter 
PII: S1383-5718(01)00174-7

NNAL has been determined to be a rodent carcinogen [3,4]. NNK and NNAL can be pyridine N-oxidized to form NNK-N-oxide and NNAL-N-oxide, respectively, which are non-genotoxic; in addition NNAL may be conjugated to form NNAL–glucuronide, which is non-genotoxic and excreted in urine. NNK and NNAL can also be α-hydroxylated at both methyl and methylene carbons adjacent to the nitroso group to generate electrophiles that can react with biological macromolecules, including DNA and proteins [5]. Adenosine dinucleotide phosphate (ADP) adducts of NNK and NNAL have been identified in studies of NNK metabolism with rat liver [6,7], however, these adducts have only been observed in vitro. Recent in vitro studies report that human buccal mucosa predominantly reduces NNK to NNAL (95–99%), in addition to metabolism by α-hydroxylation (0.6–3.8%) and pyridyl N-oxidation (0.3–2.2%) [8].

Some of the components of cigarette smoke are recognized as either substrates or competitive inhibitors of the cytochrome P450 enzymes. For example, Murphy and Heiblum [9] demonstrated that the metabolism of NNK and NNN was inhibited by the presence of nicotine, and Lee et al. [10] demonstrated that the mutagenicity of NNK was significantly inhibited by the aqueous extract of tobacco smoke and pyridine alkaloids in cigarette smoke, such as nicotine, cotinine, and nornicotine. Brown et al. [11] demonstrated that mainstream cigarette smoke significantly inhibited the formation of keto and hydroxy acid in the A/J mouse, while NNAL formation was significantly increased. These results led to the hypothesis that the mutagenicity of NNAL might be reduced in the presence of nicotine and other structurally similar pyridine alkaloids. The purpose of this study was to evaluate the mutagenic activity of NNAL in the Ames *Salmonella*/microsome assay system, and to test the inhibitory effect of tobacco smoke and its constituents, nicotine and cotinine, on the mutagenic activity of NNAL.

## 2. Materials and methods

### 2.1. Chemicals

Professor Peter Crooks of the University of Kentucky kindly prepared NNAL. Nicotine (>98% pure) and cotinine (>98% pure) were purchased from Sigma (St. Louis, MO). Aroclor-induced Hamster liver S9 was from Moltox (Boone, NC).

### 2.2. Preparation of aqueous cigarette smoke extract (ACTE)

Kentucky reference cigarettes (1R4F) obtained from the Tobacco and Health Research Institute (Lexington, KY) were smoked under the FTC protocol, and mainstream particulate matter collected on Cambridge filters. Potassium phosphate buffer (0.05 M, pH 7.0) was added to the filter pads at a ratio of 10 mg total particulate matter per milliliter. Extraction was conducted by macerating the Cambridge pads with a Stomacher lab-blender (Tekmar; Cincinnati, OH) for 20 min and then filtering the slurries with 0.45 μm membrane filters. ACTE was prepared at the concentration of 10 mg "tar" per milliliter; hence, 20 μl of ACTE represented the water-soluble constituents from 200 μg of "tar". The resulting ACTE was stored at −70°C until use.

### 2.3. Ames/Salmonella mutagenicity assays

The microsuspension assay was conducted following modifications of the Ames assay by Kado et al. [12]. Briefly, *Salmonella typhimurium* culture TA1535 was grown overnight at 37°C in Oxoid nutrient broth. Cells were harvested by centrifugation at 10,000 rpm for 10 min and re-suspended in 0.02 M phosphate buffer (pH 7.4) to yield a five-fold cell concentration. NNAL was dissolved in water instead of DMSO to avoid its inhibitory effect on the bioacitvation of NNAL [13,14]. The mutagenic activity of NNAL was tested at various S9 concentrations using either rat or hamster S9.

### 2.4. Cell survival test

Overnight cultures of *Salmonella* tester strain TA1535 were diluted to $\sim 3 \times 10^3$ cells/ml with Oxoid nutrient broth. Diluted cell suspension (0.1 ml) was combined with 0.5 ml of S9 mix and 100 μl of test articles in a test tube. A 2 ml quantity of molten top agar containing 0.5 mM histidine/biotin was added, and after mixing, the contents were poured onto minimal-glucose agar plates. Plates were incubated for 48 h at 37°C and counted for surviving colonies.

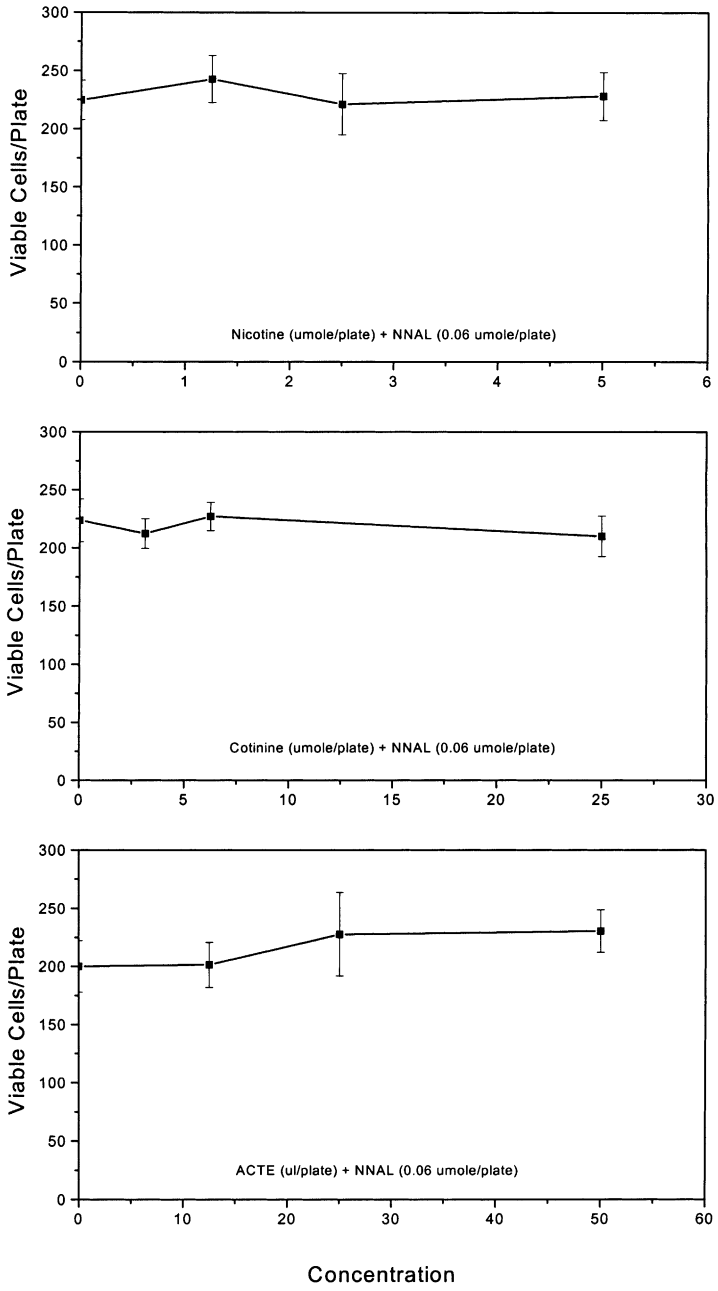


Fig. 1. Overnight cultures of *Salmonella* were diluted to $\sim 3 \times 10^3$ cells/ml with Oxoid nutrient broth and diluted cell suspensions were combined with S9 mix and test articles. A 2 ml quantity of molten top agar containing 0.5 mM histidine/biotin was added, and after mixing, the contents were poured onto minimal glucose agar plates. Plates were incubated for 48 h at 37°C and counted for surviving colonies.

## 3. Results

### 3.1. Cell survival tests

Results of cell survival tests are shown in Fig. 1. As can be seen from the data, the NNAL concentration of 0.06 μmol per plate was not cytotoxic. When combinations of NNAL and either nicotine, cotinine, or ACTE were tested, again no cytotoxicity was observed.

### 3.2. Mutagenicity assays of NNAL

The mutagenicity of NNAL was tested using both rat and hamster S9 (5 and 10%, v/v) in microsuspension assays (Fig. 2). The data presented indicate that NNAL was mutagenic in the presence of either rat or hamster S9, with the highest response obtained in the presence of 10% hamster S9. Based on these results, tests of the inhibitory effects of various agents on NNAL mutagenicity were conducted using 10% hamster S9 and 0.06 μmol NNAL per plate.

### 3.3. Effects of nicotine on the mutagenicity of NNAL

The effects of nicotine on the mutagenicity of NNAL were tested using 0.06 μmol of NNAL and nicotine concentrations of 0–10 μmol per plate. As shown in Fig. 3, nicotine effectively reduced the number of revertants induced by NNAL. As can be seen from the graph, there is a dose-dependent response up to 1 μmol of nicotine. The cell survival test indicated no cell killing by either NNAL alone or in combination with nicotine, indicating that the reduction of revertant numbers is not due to cytotoxicity.

### 3.4. Effects of cotinine on the mutagenicity of NNAL

Cotinine, in the concentration range of 2.5–25 μmol per plate, was tested for its effects on the mutagenicity of NNAL (0.06 μmol per plate) in a microsuspension assay. The results (Fig. 4) indicated that even the lowest concentration of cotinine evaluated, i.e. 2.5 μmol per plate, achieved almost complete inhibition of NNAL mutagenicity. The cell survival test indicated no cell killing by the combination of cotinine and NNAL, indicating that the reduction of revertant numbers is not due to cytotoxicity.

### 3.5. Effects of ACTE on the mutagenicity of NNAL

ACTE tested at volumes ranging from 0 to 20 μl per plate was not mutagenic. ACTE was then tested

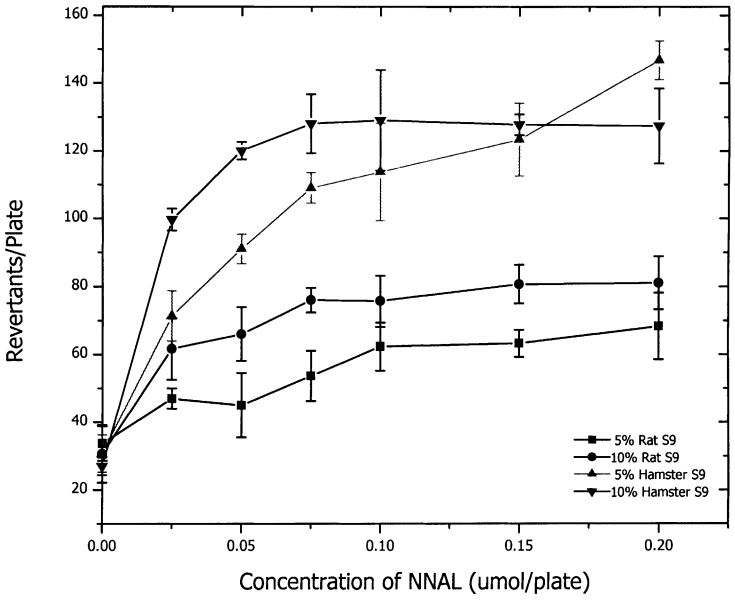


Fig. 2. The mutagenicity of NNAL was tested using both rat and hamster S9 at 5 and 10% (v/v) in microsuspension assays.

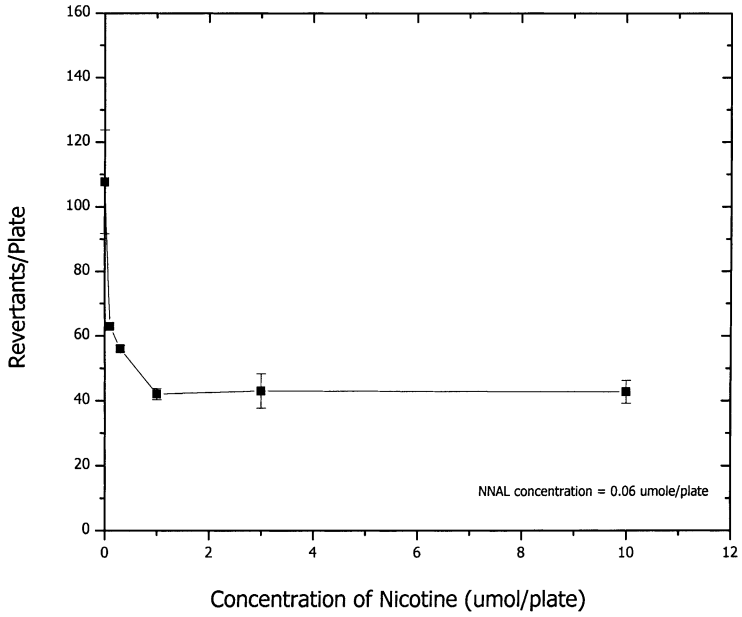


Fig. 3. The effect of nicotine on the mutagenicity of NNAL (0.06 μmol per plate) using nicotine in the concentration range of 0–10 μmol per plate with hamster S9.

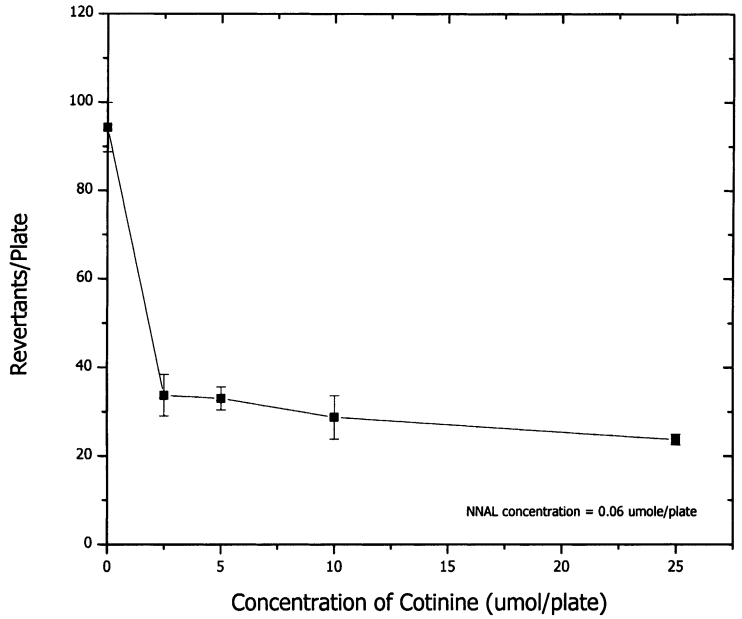


Fig. 4. The effect of cotinine on the mutagenicity of NNAL (0.06 μmol per plate) using cotinine in the concentration range of 10–100 μmol per plate with hamster S9.

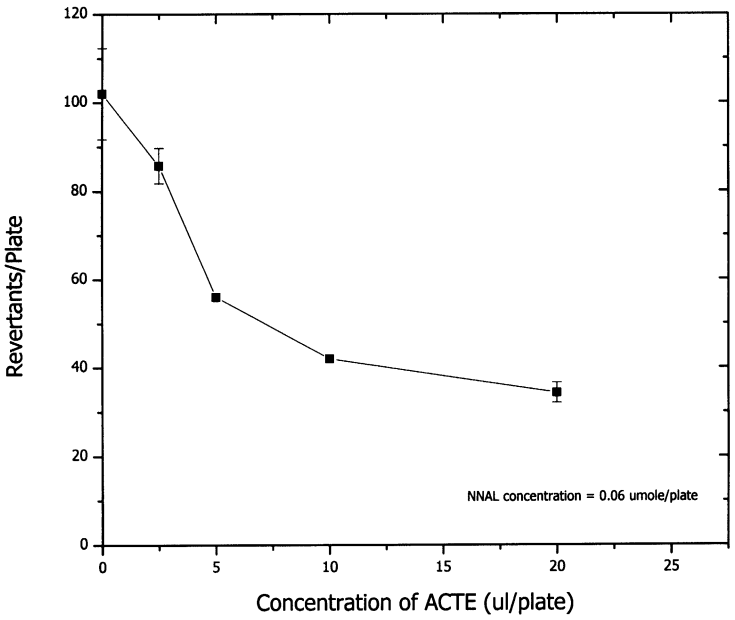


Fig. 5. The effect of ACTE on the mutagenicity of NNAL (0.06 μmol per plate) using ACTE in the concentration range of 2.5–50 μl per plate with hamster S9.

at the above volume ranges for its effect on the mutagenicity of NNAL (0.06 μmol per plate). The results (Fig. 5) indicate a dose-dependent inhibition of mutagenicity. As indicated by the cell survival test, the combination of NNAL and ACTE did not cause a decrease in cell number, indicating that the reduction of revertant numbers is not due to cytotoxicity.

## 4. Discussion

In this study, we have established that NNAL is mutagenic in the Ames/*Salmonella* mutagenicity assay in the presence of S9. Using this assay system, we monitored the inhibitory effect of ACTE and two of the pyridine alkaloids in tobacco smoke, nicotine and cotinine. All three of the test agents inhibited the mutagenicity of NNAL.

Nicotine is present at concentrations 3000–30,000-fold higher than NNK in mainstream and sidestream smoke [15–17]. Given the overwhelming concentrations of pyridine alkaloids in smoke relative to NNK, as well as the additional inhibitory smoke constituents relative to nitrosamines, the material balance in tobacco smoke is in favor of significant inhibition of metabolic activation of *N*-nitrosamines. In the present study, nicotine and cotinine were inhibitory at concentrations of 0.1 and 2.5 μmol per plate, respectively. These concentrations represent 1.7- and 41.7-fold, respectively, of the NNAL concentration (0.06 μmol per plate) in the assay systems. ACTE was inhibitory at a concentration of 2.5 μl per plate.

4-Hydroxy-1-(3-pyridyl)-1-butanone (HPB)-releasing hemoglobin adducts of NNK and NNN are often used as molecular biomarkers of tobacco-specific nitrosamine (TSNA) uptake and metabolic activation [18–21]. The results of HPB adduct analysis show only approximately a two-fold higher level in smokers compared to non-smokers [18,21] in spite of the at least two orders of magnitude greater absorption of TSNA in smokers than in non-smokers based on plasma cotinine levels [22,23]. In fact, Atawodi et al. [24] recently reported hemoglobin adduct levels in smokers (26 ± 12 fmol HPB/g globin) and non-smokers (19 ± 8 fmol HPB/g globin). Thus, it would appear that HPB-releasing hemoglobin

adducts have limited utility as biomarkers of exposure to TSNA in smokers; this based on the fact that adduct levels are frequently not much higher than assay background amounts [25]. The data presented in this paper suggest that some element of modulation plays a significant role in smokers. We have shown in previous studies [10,26] that tobacco smoke and pyridine alkaloids (nicotine, cotinine, and nornicotine) contribute to a decreased level of molecular biomarkers for NNK. Our current results using NNAL are similar to our results examining NNK inhibition, and predict a reduction in DNA methylation and DNA pyridyloxobutylation by NNAL in smokers differing from the NNAL concentration alone.

ACTE effectively inhibited NNAL mutagenicity. The specific agents in ACTE responsible for the inhibition of mutagenicity have not been identified. However, neither nicotine nor cotinine appears to be totally responsible for the inhibitory effects of ACTE. The nicotine content of ACTE prepared from 1R4F cigarettes was 0.54 μmol/100 μl. The equivalent concentrations of nicotine from ACTE used in this study would be 0.0135–0.108 μmol per plate. Direct exposure of nicotine at a concentration of 0.3 μmol per plate was needed to achieve a comparable degree of inhibition that was induced by ACTE. Realistically, the nicotine content in ACTE is too low to account for the degree of inhibition realized; hence, it appears that ACTE contains, in addition to nicotine, water-soluble smoke constituents that inhibit the mutagenicity of NNAL.

Plants contain a wide array of antimutagens [27], and several studies have shown a number of agents occurring in plants to be antimutagenic and antitumorigenic against *N*-nitrosamines. Plant polyphenolic compounds have been demonstrated to exert antimutagenic effects against NNK [28]. Betel leaf extract was likewise found to be antimutagenic to both NNK and NNN, as tested by the Ames/*Salmonella* mutagenicity assay [29]. Finally, a series of isothiocyanates [30], as well as ellagic acid [31], butylated hydroxyanisole [31], indole-3-carbinol [32], D-limonene [33], diallyl sulfide [34], and green tea extract [35,36] have been shown to inhibit the metabolic activation of nitrosamines, as well as their tumorigenesis, in the lungs of rats and mice. Thus, it is not surprising that the complex matrix of tobacco smoke [37] includes chemicals that could inhibit nitrosamines mutagenicity.

The concentrations of nicotine and nitrosamines in tobacco smoke vary among different cigarettes [38], but nicotine is present in concentrations 3000–30,000-fold higher than NNK in mainstream and sidestream smoke [15–17]. In view of the overwhelming concentration of pyridine alkaloids relative to nitrosamines, as well as the additional constituents in tobacco smoke, the activation of *N*-nitrosamines to biologically active species was expected to be significantly inhibited. Moreover, only if the metabolically active forms of cytochromes P450 were appreciably saturated by nicotine, cotinine, and other tobacco smoke components would there be significant inhibition of the metabolism of NNK or NNAL. A previous study [26] was designed to maximize for lung DNA adduct formation in A/J mice as illustrated by the fact that the doses of NNK (3.75 and 7.5 μmol per mouse) administered were doses that induced lung adenoma [26,39]. These doses of NNK are equivalent to the amount of NNK extracted from the mainstream smoke of 10,000–20,000 cigarettes and were administered in one single injection. Smoke concentrations were 0.4, 0.6, and 0.8 mg WTPM/L of air, therefore, the amount of smoke that the animals received contained between 2.85 and 5.71 ng NNK. Based on in-house physiological studies, the maximum amount of cigarette smoke that an A/J mouse would be exposed to in 2 h would be equivalent to less than one cigarette. Therefore, the smoke of less than one cigarette inhibited the biological effects of the NNK from several thousand cigarettes and none of the cigarette smoke concentrations alone induced any lung DNA adducts formation. However, when mice were co-administered cigarette smoke and NNK, lung DNA adduct levels were significantly ($P < 0.05$) reduced by 46% as compared to animals exposed to NNK only. The evidence of this study indicated that cigarette smoke statistically significantly ($P < 0.05$), not completely, inhibited the formation of lung DNA adducts-induced by exaggerated doses of NNK. Due to modulating agents in cigarette smoke, such as those described in the present study, the biologically relevant dose of NNAL from cigarette smoking is likely to be much lower than predicted from studies comparing the biological activity of pure NNAL with plasma concentration of NNAL.

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