Loma Linda University

TheScholarsRepository@LLU: Digital Archive of Research, Scholarship & Creative Works

Loma Linda University Electronic Theses, Dissertations & Projects

6-1994

Inhibition of NNK Mutagenesis and Metabolism by Chemopreventive Phytochemicals

Cecil Henry Miller

Follow this and additional works at: https://scholarsrepository.llu.edu/etd

Part of the Physiology Commons

Recommended Citation

Miller, Cecil Henry, "Inhibition of NNK Mutagenesis and Metabolism by Chemopreventive Phytochemicals" (1994). *Loma Linda University Electronic Theses, Dissertations & Projects*. 1714.
https://scholarsrepository.llu.edu/etd/1714

This Dissertation is brought to you for free and open access by TheScholarsRepository@LLU: Digital Archive of Research, Scholarship & Creative Works. It has been accepted for inclusion in Loma Linda University Electronic Theses, Dissertations & Projects by an authorized administrator of TheScholarsRepository@LLU: Digital Archive of Research, Scholarship & Creative Works. For more information, please contact scholarsrepository@llu.edu.

LOMA LINDA UNIVERSITY

Graduate School

INHIBITION OF NNK MUTAGENESIS AND METABOLISM BY CHEMOPREVENTIVE PHYTOCHEMICALS

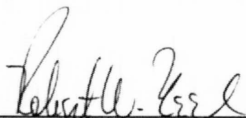
by

Cecil Henry Miller

A Dissertation in Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy in Physiology

June 1994

Each person whose signature appears below certifies that this dissertation in his opinion is adequate, in scope and quality, as a dissertation for the degree Doctor of Philosophy.



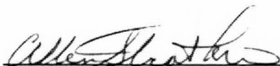
, Chairman

Robert W. Teel, Professor of Physiology

Raymond G. Hall, Associate Professor of Physiology

Benjamin H.S. Lau, Professor of Microbiology

George Maeda, Assistant Professor of Physiology



Allen Strother, Professor of Pharmacology

ACKNOWLEDGEMENTS

I am grateful to the Department of Physiology (Loma Linda University, Loma Linda, CA) for providing teaching and research assistantships. I also am thankful for the Selma Andrews Scholarship from the Graduate School (Loma Linda University).

TABLE OF CONTENTS

LIST OF FIGURES vi
LIST OF TABLES ix

I. INTRODUCTION 1

A. References 17

II. OPTIMIZING CONDITIONS FOR NNK-INDUCED MUTAGENESIS USING THE AMES/SALMONELLA/MICROSOME ASSAY 24

A. Summary 26
B. Introduction 28
C. Materials and Methods 34
D. Results 37
E. Discussion 59
F. References 71

III. MODULATION OF THE MUTAGENICITY AND METABOLISM OF THE TOBACCO-SPECIFIC NITROSAMINE 4-(METHYLNITROSAMINO)-1-(3-PYRIDYL)-1-BUTANONE (NNK) BY PHENOLIC COMPOUNDS 82

A. Summary 84
B. Introduction 86
C. Materials and Methods 89
D. Results 95
E. Discussion 117
F. References 124

IV. EFFECTS OF COMPOUNDS OF PLANT ORIGIN ON THE MUTAGENICITY AND METABOLISM OF THE TOBACCO-SPECIFIC NITROSAMINE NNK 135

A. Summary 137
B. Introduction 139
C. Materials and Methods 141

D. Results....146
E. Discussion....155
F. References....161

V. EFFECTS OF CAPSAICIN ON LIVER MICROSOMAL METABOLISM OF THE TOBACCO-SPECIFIC NITROSAMINE NNK....171

A. Summary....173
B. Introduction....175
C. Materials and Methods....178
D. Results and Discussion....182
E. References....195

VI. DISCUSSION....200

A. References....204

LIST OF FIGURES

Figure | Page

Chapter I

1. Mechanism for oxygenation of substrate by cytochrome P450 isozymes 6

2. Pathways of NNK metabolism 9

3. Multistage process of carcinogenesis 12

Chapter II

1. The effect of master plate age on mutagenesis in *Salmonella typhimurium* TA1535 38

2. The effect of master plate age on mutagenesis in *Salmonella typhimurium* TA100 40

3. The effect of agar plate age on mutagenesis in *Salmonella typhimurium* TA1535 42

4. The effect of agar plate age on mutagenesis in *Salmonella typhimurium* TA100 44

5. Comparison of NNK-induced mutagenesis in *Salmonella typhimurium* TA1535 with NNK dissolved in saline and in DMSO 46

6. Effect of DMSO on NNK-induced mutagenesis in *Salmonella typhimurium* TA1535 using ISF, MC and PB-induced rat liver S9 48

7. Effect of DMSO on NNK-induced mutagenesis in *Salmonella typhimurium* TA1535 using AR, DM and ET-induced rat liver S9 50

8. Toxicity of DMSO on *Salmonella typhimurium* TA1535 52

9. Dose response of NNK-induced mutagenesis in *Salmonella typhimurium* TA1535 using ISF, MC and PB-induced rat liver S9 54

10. Dose response of NNK-induced mutagenesis in *Salmonella typhimurium* TA1535 using AR, DM and ß-NF-induced rat liver S9 56

11. Dose response of different concentrations of hamster microsomes on NNK-induced mutagenesis in *Salmonella typhimurium* TA1535 58

Chapter III

1. Immunoblots of P450 monooxygenases from control, PB and ß-NF-induced hamster liver microsomes 97

2. Molecular structures of phenolic compounds 98

3. Effect of phenolic compounds on the NNK-induced mutagenesis in *Salmonella typhimurium* TA1535 using noninduced hamster liver microsomes 101

4. Effect of phenolic compounds on the NNK-induced mutagenesis in *Salmonella typhimurium* TA1535 using PB-induced hamster liver microsomes 103

5. Effect of phenolic compounds on the NNK-induced mutagenesis in *Salmonella typhimurium* TA1535l using ß-NF-induced hamster liver microsomes 105

6. Effect of PB and ß-NF treatment on the NNK-induced mutagenesis in *Salmonella typhimurium* TA1535 using hamster liver microsomes 107

7. Metabolic pathways of NNK 109

Chapter IV

1. Effect of different concentrations of five phytochemicals on NNK-induced mutagenesis in *Salmonella typhimurium* TA1535 using noninduced hamster liver microsomes 148

2. Metabolic pathways of NNK 150

3. Effects of five phytochemicals on the metabolism of NNK by noninduced hamster liver microsomes 154

Chapter V

1. Metabolic pathways of NNK 184

2. Metabolism of NNK by liver microsomes from control, PB and ß-NF-induced hamsters 187

3. Effect of concentration of capsaicin on the metabolism of NNK by saline-injected hamster liver microsomes 189

4. Effect of capsaicin (0.5 mM) on the metabolism of NNK by hamster liver microsomes 192

LIST OF TABLES

Table Page

Chapter III

1. Effects of saline, phenobarbitol, and ß-naphthoflavone treatment on the metabolism of NNK by hamster liver microsomes.....111

2. Effects of phenolic compounds (0.4 umol) on the metabolism of NNK by liver microsomes from saline-treated hamsters.....112

3. Effects of phenolic compounds (0.4 umol) on the metabolism of NNK by liver microsomes from phenobarbitol-induced hamsters.....114

4. Effects of phenolic compounds (0.4 umol) on the metabolism of NNK by liver microsomes from ß-napthoflavone-induced hamsters.....116

Chapter IV

1. Effects of phytochemicals on the metabolism of NNK by hamster liver microsomes.....152

Chapter V

1. A comparison of the metabolism of NNK by liver microsomes from saline-injected, phenobarbitol and ß-naphthoflavone-induced hamsters.....186

2. Effect of concentration of capsaicin on the metabolism of NNK by liver microsomes from saline-injected hamsters.....190

3. Effect of capsaicin on the metabolism of NNK by hamster liver microsomes.....193

CHAPTER I

INTRODUCTION

Tobacco products have been used by man for many centuries. Mayan carvings from 600-900 A.D. display figures of individuals chewing tobacco leaves. Archaeologists have found seeds from *Nicotiana* plants in their excavations of early civilizations from Mexico and Peru [Davis, 1988]. When Europe discovered the new world, international trade between America and Europe began. One of the first products European traders obtained from native Americans was tobacco. By the 1600's smoking had evolved from the American natives smoking their peace pipes to a world wide pastime [IARC, 1986]. In 1699, 4.5 tons of tobacco were imported into England [Redmond, 1970]. Tobacco production continued to increase and by 1982, 6.7 million tons of tobacco were produced in America alone, with an average per person yearly consumption of 3500 cigarettes [IARC, 1986]. There are currently an estimated 56 million smokers and over 12 million smokeless tobacco users in the USA [Weinstein 1991; Hecht and Hoffman, 1988]. The American Cancer Society estimates that cigarette smoking contributed to more than 157,000 cancer deaths in America in 1991 [Shopland et al., 1991].

The earliest suspicion that there could be a link between the use of tobacco products and cancer was in 1761 when John Hill suggested

that tobacco use in the form of snuff may result in the development of nasal cancers [Redmond, 1970]. This report appeared nineteen years before Percival Pott reported that the high incidence of scrotal cancer in the chimney sweeps was possibly due to their exposure to carcinogens in the soot from chimneys [Sirica, 1989]. The search for the chief active ingredient of tobacco began with the discovery of an "essential oil" in 1807 by Cerioli. The presence of this active substance was found in 16 species of *Nicotiana* plants and by 1832 the empirical formula of nicotine was determined [Holmstedt, 1988]. During the last several decades research endeavors have investigated the physiological responses to nicotine. Nicotine is the critical pharmacologic agent that is responsible for the addiction associated with tobacco use [US Surgeon General, 1988]. It induces a variety of stimulant and depressant effects involving many physiologic systems including the nervous, endocrine, cardiovascular, and gastrointestinal systems. The effects of this drug are at least in part responsible for the addiction, development of tolerance and for the withdrawal symptoms associated with the cessation of tobacco use [Hecht et al., 1991].

At least 4000 different compounds from tobacco smoke have been characterized. Of these, over 40 are carcinogenic in laboratory animals [Hoffman and Hecht, 1990; Roberts, 1988]. The polycyclic aromatic hydrocarbons and the nitrosamines are the major carcinogens involved in cigarette smoking induced lung cancer [Hoffmann and Hecht, 1985]. Nicotine itself is not carcinogenic. It is,

however, a precursor to the powerful carcinogenic nitrosamines that are found in tobacco and tobacco smoke. The tobacco-specific nitrosamines (TSNS) are products of nicotine nitrosation. These nitrosation reactions occur during tobacco processing, tobacco chewing and cigarette smoking [Hecht et al., 1983; Hecht and Hoffmann, 1988]. The primary TSNS produced from the nitrosation of nicotine are a nicotine-derived nitrosamino ketone, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK); a nitrosamino aldehyde, 4-(methylnitrosamino)-4-(3-pyridyl)-butanal (NNA); and a demethylated form of NNA, N-nitrosonornicotine (NNN) [Hecht and Hoffmann, 1988; Hecht et al., 1978]. The most prevalent TSNS in tobacco and tobacco smoke are NNK and NNN. The aldehyde NNA is not easily detected but products of its reduction to an alcohol derivative, iso-NNAL, and a carboxylic acid derivative, iso-NN-acid, are found in tobacco products [Brunnemann et al., 1987]. Studies of carcinogenicity in animals have demonstrated that NNK selectively induces lung tumors in rats, mice and hamsters, irrespective of route of administration [Hecht and Hoffmann, 1988]. The doses of NNK and NNN that produce tumors in experimental animals are approximately the same as the exposure a smoker receives if they smoke 1 pack per day for 40 years. Similarly snuff dippers by using 10 g tobacco per day for 40 years would be exposed to approximately the same doses of NNK and NNN that cause oral cavity tumors in rats [Hecht et al., 1991].

Most chemical carcinogens must be metabolized to produce reactive products which then exert the carcinogenic effects. The metabolic activation of the carcinogens produce electrophile substrates that are highly reactive towards DNA. The DNA modifications that occur cause changes in the expression of oncogenes (increased expression) and/or tumor suppressor genes (decreased expression). These genes regulate the growth, proliferation, and differentiation pathways of cells. Altering their normal expression greatly increases the probability of neoplastic transformation and the subsequent development of cancer [Guengerich, 1991; Harris, 1991]. The metabolism of these carcinogens and other xenobiotics occur via a series of enzyme-catalyzed reactions. The primary purpose of these reactions in mammalian systems is detoxification and excretion. Most xenobiotics are lipophilic and therefore must be made polar so they can be excreted in the urine. The reactions have been classified into phase I and phase II reactions [Williams, 1978]. Phase I reactions occur in the endoplasmic reticulum and include oxidation, hydroxylation, reduction and hydrolysis. These reactions result in oxygenation of lipophilic molecules by inserting a polar functional group. This renders the chemicals more water soluble and more reactive with conjugating agents. The phase I reaction products, however, often produce substrates that are highly nucleophilic and can easily attack DNA, causing mutations. Phase II cytoplasmic enzymes conjugate these highly reactive substrates to substances like glutathione and

glucuronate. The enzyme UDP-glucuronosyltransferase catalyzes the transfer of glucuronic acid from UDP-glucuronic acid to electrophiles that contain the following functional groups: alcohol, carboxylic acid, carbamate, amine, sulfonamide, thiol, hydroxylamine, dithiocarbamate and α or ß-unsaturated ketone [Kasper and Henton, 1980; Yang and Yoo, 1991]. These reactions result in conjugation of the xenobiotic electrophiles to form glucuronides. Other electrophiles such as epoxides, alkyl and aryl halides, sulfates and 1,4-unsaturated carbonyl compounds are conjugated with glutathione. The glutathione-electrophile conjugations are catalyzed by glutathione-S-transferase [Jakoby and Habig, 1980; Yang and Yoo, 1991]. After phase II conjugations, these electrophiles have decreased biologic activity, and increased hydrophilicity thereby enhancing their excretion [Parke et al., 1991; Yang and Yoo, 1991].

The enzymes that catalyze the phase I reactions are mixed function oxidase enzymes (MFO) that are collectively called cytochrome P450 isozymes. These P450 enzyme systems are composed of two enzymes, P450 and NADPH-P450 reductase, both of which are integral membrane proteins (Figure 1). The NADPH reductase is a protein containing FMN and FAD cofactors. The reductase transfers electrons from NADPH to the cytochrome P450 via the oxidation and reduction of the FMN and FAD cofactors. Cytochrome b_5 can also shuttle electrons from NADH to the cytochrome P450 [Nebert and Gonzalez, 1987; Parke et al., 1991; Guengerich, 1993]. The cytochrome P450 protein is a heme

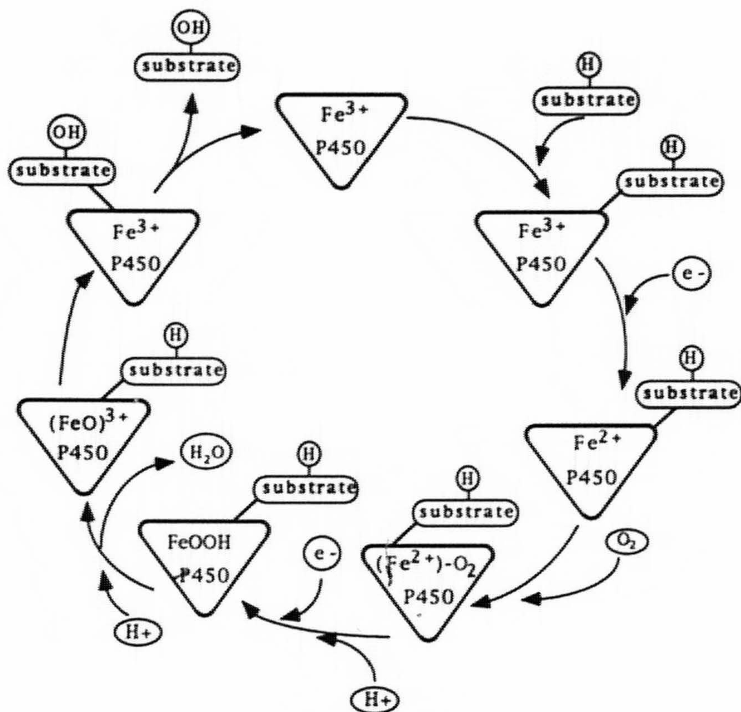
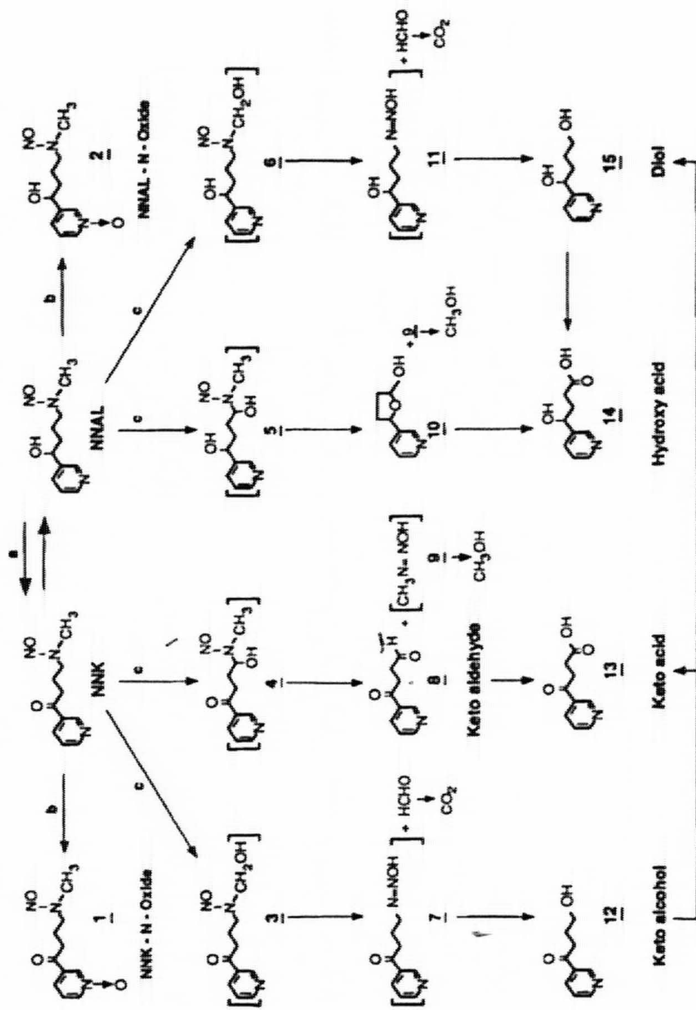


Figure 1: Catalytic action of cytochrome P450 isozymes. Cyclic mechanism of substrate oxygenation by cytochrome P450 enzymes. (Adapted from Guengerich, 1993)

containing protein that can easily be oxidized or reduced. The catalytic activity of the P450 system is as follows (Figure 1). The substrate (chemical) binds to the P450 at a specific binding site just above the heme group. This binding can occur only when the heme iron is in the oxidized (Fe^{+3}) form. An electron from the reductase or cytochrome b_5 is then transferred to the iron atom reducing it to the (Fe^{+2}) state. The reduced P450 can then bind molecular O_2 to a specific O_2 binding site at the heme. A second electron and a proton (H^+) are then added to the reduced iron, forming a FeOOH complex. With the loss of water, a $(FeO)^{+3}$ complex is produced. This complex is a very potent oxidizing agent and transfers its unstable oxygen to the substrate, oxidizing it. The oxidized substrate is then released and the cytochrome P450 can repeat the cycle with another substrate [Guengerich, 1993; Parke et al., 1991]. These oxidized substrates have several possible fates in the cell. They may be conjugated by phase II enzymes and subsequently excreted as described above or they may form DNA adducts and potentially DNA mutations.

NNK, like most carcinogens, is relatively inert biologically until it is metabolized by the P450 isozymes. Several of the metabolic products produce reactive species that either form DNA adducts or cause nucleotide alteration [Murphy et al., 1990]. The metabolism of NNK in hepatic microsomes is shown in Figure 2. There are three types of metabolic pathways for NNK. They are carbonyl reduction to NNAL (pathway a); N-oxidation of the pyridine rings of NNK and

Figure 2: Metabolic pathways of NNK: carbonal reduction (pathway a), pyridine N-oxidation (pathway b), and α-carbon hydroxylation (pathway c).
Metabolites: NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol;
NNK-N-oxide, 4-(methylnitrosamino)-1-(3-pyridyl-N-oxide)-1-butanone;
NNAL-N-oxide, 4-(methylnitrosamino)-1-(3-pyridyl-N-oxide)-1-butanol;
keto alchol, 4-oxo-4-(3-pyridyl)-1-butanol;
hydroxy acid, 4-hydroxy-4-(3-pyridyl) butyric acid;
keto aldehyde, 4-oxo-4-(3-pyridyl) butanal;
keto acid, 4-oxo-4-(3-pyridyl) butyric acid;
diol, 4-hydroxy-1-(3-pyridyl)-1-butanol.
The structures in brackets are hypothetical intermediates.
(Adapted from Jorquera et al., 1992; Hecht et al., 1980).


NNK
NNAL
NNK - N - Oxide
NNAL - N - Oxide
Keto aldehyde
Keto alcohol
Keto acid
Hydroxy acid
Diol
+ HCHO
CO_2
CH_3OH
a
b
c

NNAL yielding the N-oxides NNK-N-oxide and NNAL-N-oxide (pathway b) and the α-hydroxylation of either the methyl or methylene carbon adjacent to the N-nitroso nitrogen (pathway c). Hydroxylation of the methyl group gives the α-hydroxynitrosamine 3, which is unstable and spontaneously decomposes to 4-(3-pyridyl)-4-oxobutyldiazohydroxide and formaldehyde (7). This oxobutylating intermediate can undergo denitrosation and be hydrolyzed to a keto alcohol (12). Hydroxylation of the methylene group (carbon 4) gives the α-hydroxynitrosamine intermediate 4, which yields 4-oxo-4-(3-pyridyl)-butanal, a ketoaldehyde 8 and a methyldiazohydroxide 9. The subsequent oxidation/reduction of the ketoalcohol and ketoaldehyde yields the secondary α-carbon hydroxylation metabolites ketoacid 13, hydroxyacid 14 and diol 15. The α-hydroxylation of the methylene (carbon 4) of NNAL yields the hydroxy acid 14 and a methyl diazohydroxide 9 [Hecht et al., 1980; Jorquera et al., 1992]. NNN metabolism produces the same oxobutylating intermediate 7 as seen with NNK metabolism [Pool-Zobel et al., 1992]. Of these metabolites only two are genotoxic. The oxobutylating intermediate 7 forms adducts with DNA and hemoglobin [Murphy et al., 1990]. The methyldiazohydroxide 9 methylates amino acids and produces the following nucleotide mutations 7-methylguanine, O^6-methylguanine and O^4-methylguanine [Murphy et al., 1990]. These mutations are known to play active roles in the chemical carcinogenesis of NNK [Rivenson et al., 1991].

Carcinogenesis is a multistage process which is driven by genetic and epigenetic damage to susceptible cells. Figure 3 outlines some of the basic factors that participate in this process. The first steps of the carcinogenic process is the exposure of normal cells to chemical, physical or microbial carcinogens that result in genetic changes that make the cell less responsive to regulators such as negative growth factors, terminal cell differentiators, and/or agents of apoptosis. These initiated cells then proliferate to a greater extent than normal cells. This proliferation is called promotion. During promotion the initiated cells exhibit clonal expansion and are gradually converted into malignant cells by activation of protooncogenes and/or inactivation of tumor suppressor genes. These malignant cells continue to have phenotypic changes and express genomic instability that is manifested by abnormal numbers and structure of chromosomes, gene amplification and altered gene expression [Harris, 1991].

The process of carcinogenesis can be inhibited. Current estimates indicate that 50-80% of human cancer is potentially preventable [Weinstein, 1991]. Cancer prevention which utilizes chemical compounds as the preventive agents is called chemoprevention. Extensive studies in both animals and humans provide evidence that dietary factors play an important role in cancer prevention. Many of these studies have investigated the role that nutrient components like dietary fat, minerals and vitamins play in cancer prevention. Other studies have concentrated on carcinogens like aflatoxin,

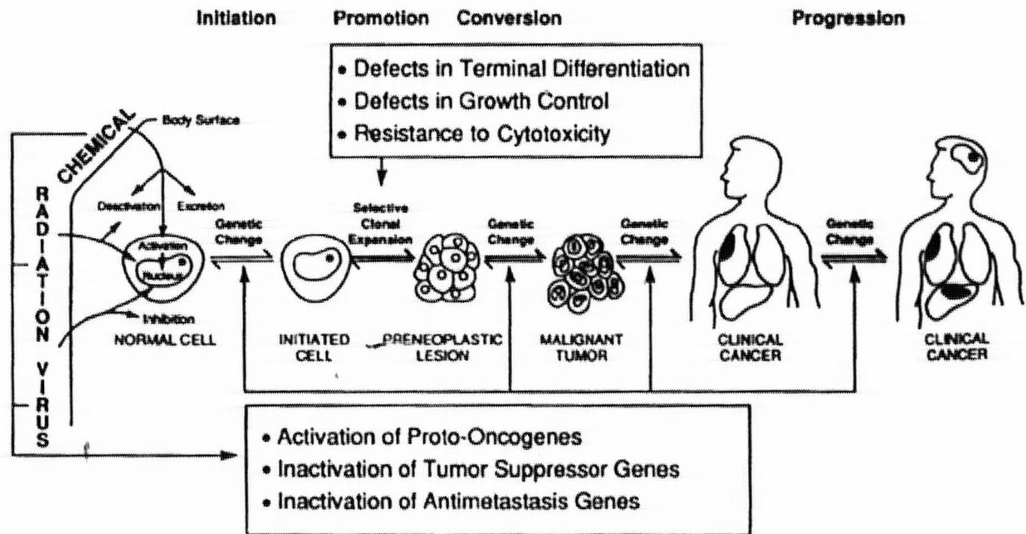


Figure 3: Carcinogenesis is a multistage process involving multiple genetic and epigenetic events in protooncogenes, tumor suppressor genes, and anti-metastasis genes. (Adapted from Harris, 1991)

nitrites, MeIQ, etc. that are found in food products [Weinstein, 1991]. Many current studies are being done which investigate the effect nonnutrient food components have on cancer prevention. These studies were pioneered by Silverstone [1952] who demonstrated that animals fed a crude diet had a lower cancer risk than animals fed a semipurified diet. The crude diets (purina chow) contain components such as grain, beet pulp, alfalfa meal, cane molasses, fishmeal, and other crude products. The semipurified diets consisted of defined constituents such as casein-protein, starch-carbohydrate, corn oil-fat, minerals and vitamins. Even though both diets had approximately the same calories and same composition of nutrients (carbohydrate, protein, fat, etc.), the animals on the semipurified diet had a much higher (2-5x) risk for cancer than those animals on the crude diet [Silverstone, 1952; Wattenberg, 1992a]. Similar studies have been done recently where groups of animals were fed either a crude or semipurified diet for two weeks before a carcinogenic challenge was given. The same diets were continued after the carcinogen challenge. In one study when NNK was given, the animals on the semipurified diet had 3 times as many pulmonary adenomas as those animals on the crude diets [Hecht et al., 1989]. The search for the plant chemicals (phytochemicals) that were responsible for this chemoprevention began as a result of these types of experiments.

Lee Wattenberg [1992b] has classified chemopreventive agents into two categories, blocking agents and suppressing agents. Blocking agents have a barrier function. They inhibit initiators and/or

promotors. Blocking can occur by preventing carcinogen activation (phase I enzymes), by enhancing detoxification (phase II enzymes) and by trapping reactive carcinogenic species. Many blocking agents inhibit tumor promoters by preventing oxygen radicals from forming or scavenging the radicals that do form. Suppressing agents inhibit the process of promotion during which the initiated cells become transformed into malignant cells. These cells have already been exposed to doses of carcinogens that will cause them to ultimately progress to malignancy. Suppressing agents work during the earliest stages of cancer development [Fearson and Vogelstein, 1990]. Since genetic damage has already been done, suppressing agents must be chronically administered. Suppression is maintained only as long as the suppressing agent is present; upon removal, the initiated neoplastic process continues.

In recent years phytochemicals and their potential for chemoprevention have been studied extensively. Some phytochemicals act as blocking agents that inhibit the biologic activation of induced carcinogens by inhibition of phase I (P450) enzymes. Chung et al. [1984; 1985] have studied the effects of benzyl isothiocyanate on nitrosamine metabolism in rat liver microsomes and cultured esophageal cells. They found that administration of benzyl isothiocyanate 2 hrs before sacrifice of the animals markedly inhibited the activation of NNK and N-nitrosodimethylamine [Chung et al., 1984; 1985]. Similarly Brady et al. [1988] demonstrated that diallyl sulfide inhibited microsomal

metabolism of nitrosamines by rats when administered 3 hrs before sacrifice. d-Limonene and citrus fruit oils were also found to inhibit the carcinogenicity of NNK [Wattenberg and Coccia, 1991]. In addition to blocking by inhibiting metabolism, some phytochemicals have chemopreventive blocking action by enhancing the detoxification (phase II) enzymes. Diallyl sulfide, an organosulfur compound found in *Allium* plant species (garlic and onions), was found to inhibit tumor formation in the lung, and concomitantly increased glutathione-S-transferase activity in the lung [Sparnins et al., 1988]. Aromatic isothiocyanates like benzyl isothiocyanate from cruciferous vegetables (broccoli and cabbage) also induce glutathione-S-transferase activity [Wattenberg, 1992a]. Other phytochemicals exhibit their blocking effect by inhibiting the activity of tumor promoters. These blocking agents inhibit the arachidonic acid cascade, have antioxidant activity and/or modify the cell's response to hormones or growth factors. Phytochemicals that have been implicated as blocking by these mechanisms are phenols, tannins, flavones, curcumin, and glucarates [Wattenberg, 1992a].

Suppressing agents are also found in many plants. Studies have shown that cruciferous vegetables, orange oil, and d-limonene exhibit tumor suppression [Elson et al., 1988; Wattenberg, 1983]. Plant polyphenolic compounds have suppressing activity by inhibiting the arachidonic acid cascade [Rubio, 1984; Kelloff et al., 1990]. The most studied suppressing agents have been the retenoids and the synthetic analogues of vitamin A [Zile et al., 1986;

Wattenberg, 1992a]. Some phytochemicals have both suppressing and blocking activity. Cruciferous vegetables, especially those containing benzyl isothiocyanate, are known to exhibit both activities [Wattenberg, 1985]. Citrus fruit oils and d-limonene also exhibit both blocking and suppressing activity [Wattenberg, 1983; Elson et al., 1988].

In terms of chemoprevention the best strategy appears to be to halt the carcinogenic process in its earliest stages. Minimizing exposure to carcinogens is of utmost importance. For tobacco users the best defence is to stop using tobacco products. However, since nicotine is so addictive other prevention methods require investigation. This study was initiated to pursue that goal. Chapter two describes the results of experiments that were done to optimize the conditions for NNK-induced mutagenesis in *Salmonella typhimurium* TA1535. Ten phytochemicals, of which five were phenolics, were tested for their efficacy in inhibiting the mutagenicity of NNK using the Ames assay and by determining their efficacy in inhibiting the microsomal metabolism of NNK. These results are presented in Chapters 3 and 4, respectively. One phytochemical, capsaicin, was then selected for more detailed studies of its effects on the metabolism of NNK. These results are presented in Chapter 5. Chapter 6, the final chapter, summarizes the studies described in chapters 2-5 in reference to this introduction.

REFERENCES

Brady JF, Li D, Ishizaki H, Yang CS (1988): Effect of diallyl sulfide on rat liver microsomal nitrosamine metabolism and other monooxygenase activities. Cancer Res 48:5937-5940.

Brunnemann KD, Genoble L, Hoffmann D (1987): Identification and analysis of a new tobacco-specific N-nitrosamine, 4-(methylnitrosamino)-4-(3-pyridyl)-1-butanol. Carcinogenesis 8:465-469.

Chung F-L, Juchatz A, Hecht SS (1984): Effects of dietary compounds on α-hydroxylation of N-nitrosopyrrolidine and N'-nitrosonornicotine in rat target tissues. Cancer Res 44:2924-2928.

Chung F-L, Wang M, Hecht SS (1985): Effects of dietary indoles and isothiocyanates on N-nitrosodimethylamine and 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanone-α-hydroxylation and DNA methylation in rat liver. Cancer Res 45:539-543.

Davis L (1988): Tobacco use and associated health risks. In Martin W, Van Loon G, Iwomoto E, Davis L (eds): "Tobacco smoking and nicotine, a neurological approach." New York: Plenum Press, pp 15-24.

Elson CE, Maltzman JL, Boston JL, Tabber MA (1988): Anticarcinogenic activity of d-limonene during initiation and promotion/progression stages of DMBA-induced mammary carcinogenesis. Carcinogenesis (Lond) 9:331-332.

Fearson ER, Vogelstein B (1990): A genetic model of colorectal tumorigenesis. Cell 61:759-767.

Guengerich FP (1991): Oxidation of toxic and carcinogenic chemicals by human cytochrome P-450 enzymes. Chem Res Toxicol 4:391-407.

Guengerich FP (1993): Cytochrome P450 enzymes: They defend the body against environmental pollutants, detoxify drugs and synthesize several important signaling molecules. American Scientist 81:440-447.

Harris CC (1991): Chemical and physical carcinogenesis: Advances and perspectives for the 1990's. Cancer Res 51:5023s-5044s.

Hecht SS, Castonguay A, Rivenson A, Mu B, Hoffmann D (1983): Tobacco specific nitrosamines: carcinogenicity, metabolism and possible role in human cancer. J Environ Sci Health Part C 1:1-54.

Hecht SS, Chen CB, Ornaf RM, Jacobs E, Adams JP, Hoffmann D (1978): Chemical studies on tobacco smoke. LVII. Reactions of nicotine and sodium nitrite: Formation of nitrosamines and fragmentation of the pyrrolidine ring. J Org Chem 42:72-76.

Hecht SS, Haley NJ, Hoffmann D (1991): Monitoring exposure to tobacco products by measurement of nicotine metabolites and derived carcinogens. In Groopman JP, Skipper RL (eds): "Molecular dosimetry and human cancer." Boca Raton, Florida: CRC Press, pp 325-361.

Hecht SS, Hoffmann D, (1988) : Tobacco-specific nitrosamines, an important group of carcinogens in tobacco and tobacco smoke. Carcinogenesis 9:875-884.

Hecht SS, Morse MA, Shanta A, Stoner GD, Jordan KG, Choi C-I, Chung F-L (1989): Rapid single-dose model for lung tumor induction in A/J mice by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and the effect of diet. Carcinogenesis (Lond) 18:1901-1904.

Hecht SS, Young R, Chen C-HB (1980): Metabolism in the F344 rat of 4-(N-methyl-N-nitrosamino)-1-(3-pyridyl)-1-butanone, A tobacco-specific carcinogen. Cancer Res 40:4144-4150.

Hoffman D, Hecht SS (1985): Nicotine-derived N-nitrosamines and tobacco related cancer: current status and future directions. Cancer Res 45:935-944.

Hoffmann D, Hecht SS (1990): Advances in tobacco carcinogenesis. In Cooper CS, Grover PL (eds): "Handbook of experimental pharmacology." vol. 94, Heidelberg: Springer-Verlag, p 63.

Holmstedt B (1988): Toxicity of nicotine and related compounds. In Prond RJ, Thurau K (eds): "Pharmacology of Nicotine." Washington, DC: IRL Press, pp 61-90.

IARC (1986): IARC monographs on the evaluation of the carcinogenic risk of chemicals to humans, vol. 38, tobacco smoking. Lyon, France: IARC pp 163-194.

Jakoby WB, Habig WH (1980): Glutathione transferases. In Jacoby WB (ed): "Enzymatic basis of detoxification, vol 2." New York: Academic Press, pp 63-94.

Jorquera R, Castonguay A, Schuller HM (1992): Effects of pregnancy and ethanol on the metabolism of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone by hamster liver and lung microsomes. Drug Metab Dispos 20:510-517.

Kasper CB, Henton D (1980): Glucuronidation. In Jacoby WB (ed): "Enzymatic basis of detoxification, vol 2." New York: Academic Press, pp 3-36.

Kelloff GJ, Malone WF, Boone CW, Sigman CC, Fay JR (1990): Progress applied chemoprevention research. Seminars Oncol 17:438-455.

Murphy SE, Palomino A, Hecht SS, Hoffmann D (1990): Dose-response study of DNA and hemoglobin adduct formation by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone in F344 rats. Cancer Res 50:5446-5452.

Nebert DW, Gonzalez FJ (1987): P450 Genes: Structure, evolution, and regulation. Ann Rev Biochem 56:945-993.

Parke DV, Ioannides C, Lewis DFV (1991): The 1990 Pharmaceutical Manufacturers Association of Canada keynote lecture: "The role of the cytochromes P450 in the detoxification of drugs and other chemicals". Can J Physiol and Pharmacol 69:537-549.

Pool-Zobel BL, Klein RG,Liegibel UM, Kuchenmeister F, Weber S, Schmezer P (1992): Systemic genotoxic effects of tobacco-related nitrosamines following oral and inhalational administration to Sprague-Dawley rats. Clin Investig 70:299-306.

Redmond DE (1970): Tobacco and cancer: the first clinical report, 1761. New Eng J Med 282:18-23.

Rivenson A, Hecht SS, Hoffmann D (1991): Carcinogenicity of tobacco-specific N-nitrosamines (TSNA): The role of the vascular network in the selection of target organs. CRC Crit Rev Toxicol 21:255-264.

Roberts DL (1988): Natural tobacco flavors. Recent Adv Tobacco Sci 14:49.

Rubio CA (1984): Antitumoral activity of indomethacin on experimental esophageal tumors. J Natl Cancer Inst 72:701-707.

Shopland DR, Eyre HJ, Pechacek TF (1991): Smoking-attributable cancer mortality in 1991: Is lung cancer now the leading cause of death among smokers in the United States? J Natl Cancer Inst 83:1142-1148.

Silverstone H, Solomon RD, Tannenbaum A (1952): Relative influences of natural and semipurified diets on tumor formation in mice. Cancer Res 12:750-756.

Sirica AE (1989): Chronology of significant events in the study of neoplasia. In Sirica AE (ed): "The pathobiology of neoplasia." New York: Plenum Press, pp 1-24.

Sparnins VL, Barany G, Wattenberg LW (1988): Effects of organosulfur compounds from garlic and onions on benzo(a)pyrene-induced neoplasia and glutathione-S-transferase activity. Carcinogenesis (Lond) 9:131-134.

US Surgeon General (1988): "The health consequences of smoking. Nicotine addiction." U.S. Dept. of Health and Human Services, Public Health Service, National Institutes of Health, Publication No. 88-8406.

Wattenberg LW (1983): Inhibition of neoplasia by minor dietary constituents. Cancer Res 43:2448s-2453s.

Wattenberg LW (1985): Chemoprevention of cancer. Cancer Res 45: 1-8.

Wattenberg LW (1992a): Inhibition of carcinogens by minor dietary constituents. Cancer Res 52:2085s-2091s.

Wattenberg LW (1992b): Chemoprevention of cancer by naturally occurring and synthetic compounds. In Wattenberg L, Lipkin M, Boone CW, Kelloff GJ (eds): "Cancer chemoprevention." Boca Raton, Florida: CRC Press, pp 19-39.

Wattenberg LW, Coccia J (1991): Inhibition of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone carcinogenesis in mice by d-limonene and citrus fruit oils. Carcinogenesis (Lond) 12:115-117.

Weinstein IB (1991): Cancer prevention: Recent progress and future opportunities. Cancer Res 51:5080s-5085s.

Williams RT (1978): Nutrients in drug detoxification reactions. In Hathcock JN, Coon (eds): "Nutrition and drug interrelations." New York: Academic Press, pp 303-318.

Yang CS, Yoo JSH (1991): Nutrition and xenobiotic metabolism. In Rowland IR (ed): "Nutrition, toxicity and cancer." Boca Raton, Florida: CRC Press, pp 53-91.

Zile MH, Callum ME, Roltsch IA, DeHoog JV, Welsch CW (1986): Effects of moderate vitamin A supplementation and lack of dietary vitamin A on the development of mammary tumors in female rats treated with low carcinogenic dose levels of 7,12-dimethylbenz(a)anthracene. Cancer Res 46:3495-3503.

CHAPTER II

OPTIMIZING CONDITIONS FOR NNK-INDUCED MUTAGENESIS USING THE AMES/SALMONELLA MICROSOME ASSAY

Cecil H. Miller[1], Robert W. Teel[2], and Juanita P. Moses[3]

[1]Graduate student that conducted all the experiments, collected the data and prepared the manuscript

[2]Student's research committee chairman who supervised the research and the manuscript preparation

[3]Undergraduate student who assisted with some of the experiments and data collection while participating in a summer research program

OPTIMIZING CONDITIONS FOR NNK-INDUCED MUTAGENESIS USING THE AMES/SALMONELLA MICROSOME ASSAY

C.H. Miller[a], R.W. Teel[a], and J.P. Moses[b]

[a]Department of Physiology and Pharmacology,
School of Medicine, Loma Linda University,
Loma Linda, CA 92350 (U.S.A.)
[b]Department of Chemistry, Andrews University,
Berrien Springs, MI 49104 (U.S.A.)

Key words: agar plate, master plate, age, DMSO, Ames assay

Correspondence: R.W. Teel,
Department of Physiology and Pharmacology,
School of Medicine, Loma Linda University,
Loma Linda, CA 92350 (U.S.A.)

SUMMARY

Many factors can contribute to experimental variability when using the Salmonella/mammalian microsome assay. This study was conducted to determine the conditions that would optimize NNK-induced mutagenesis. The mutagens 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and N-methyl-N-nitro-N-nitrosoguanidine (MNNG) were used with *Salmonella typhimurium* strain TA1535. Aflatoxin B_1 (AFB_1), benzo[a]pyrene (BaP), and MNNG were used with strain TA100. Metabolic activation was provided by rat liver S9 (Fisher 344, Sprague-Dawley) and Syrian hamster liver microsomes. Studies of the effect of master plate age on mutagenesis showed that NNK and MNNG-induced mutagenesis decreased when nutrient broth cultures were grown from aged master plates. AFB_1, and BaP-induced mutagenesis in strain TA100 was unaffected by age of the master plate, but MNNG-induced mutagenesis in TA100 was enhanced 3-fold by using cultures that were grown from 4 week old master plates. The age of bottom agar in prepared agar plates also affected mutagenesis. In TA1535, NNK-induced mutagenicity was enhanced 3-fold when 4 week old agar plates were used, but MNNG-induced mutagenicity decreased when older agar plates were used. Older agar plates enhanced the mutagenicity of AFB_1, BaP, and MNNG in TA100. MNNG-induced mutagenicity was most sensitive to agar plate age. DMSO used as a solvent for NNK, inhibited mutagenesis mediated by induced Fisher

rat liver S9. The relative effectiveness of the different induced fractions of S9 in activating mutagenesis in TA1535 by NNK was Arochlor > Phenobarbitol > Isosafrol > Dexamethasone > Methylcholanthrene > Ethanol. Dose reponse studies of the effect of DMSO showed that induced S9 fractions were sensitive to as little as 10 μl DMSO. Dexamethasone-induced S9 was most sensitive to DMSO. Two hundred μl DMSO in the top agar was toxic to TA1535. NNK concentration and hamster microsomal protein optimization curves were determined. Our results indicate that NNK mutagenesis can be optimized by using freshly prepared master plates and agar plates, minimizing the use of DMSO, and by using 80 mM NNK in saline with 1.0 mg hamster liver microsomal protein per plate.

INTRODUCTION

Bacterial tests for mutagenicity are the most widely used screens for genotoxicity [Gatehouse, 1987]. These tests are especially valuable for mutagenicity screening because they are rapid and inexpensive. The bacterial systems that were used before the development of the *Salmonella*/microsome assay were primarily foreward mutation assays. These assays have the disadvantage of only detecting mutagenicity for a very narrow range of substances. In one early chemical screening test, using the *Escherichia coli* streptomycin-dependent plate assay, of the 431 different substances tested, only 22 were found to be mutagenic [Szybalski, 1958]. The need to develop a more efficient and sensitive bacterial screening system was obvious. This challenge was met primarily by Dr. Bruce Ames and his colleagues with their reversion mutation assay [Ames et al., 1975; Maron and Ames, 1983]. Since reversion assays contain well defined induced mutations, there are only a few specific reverse mutations that can restore function to the inactive genes [Josephy, 1989]. This greatly enhances the specificity of the assay. It also allows one to easily determine the kind of mutation that occurred which restored the normal gene function.

Many investigators have contributed toward optimizing the sensitivity and reliability of this assay. These efforts have focused on repeatability, varations within a laboratory [Myers et al., 1987;

Cheli et al., 1980] and reproducibility, variations between different laboratories [Myers et al., 1987; Friedrich, 1982; Grafe et al., 1981]. Attempts have also been made to standardize the assay [Belser et al., 1981] and to calibrate it [Claxton et al., 1991 b,c]. Standardization of the procedure can increase reproducibility but variations in experimental results cannot be completely eliminated due to the intrinsic variability of the bacterial tester strains and mutagens [Cheli et al., 1980]. Due to this intrinsic variability, the Salmonella mutagenicity assay does not provide exact specific mutagenic activity values such as mutants per nanomole [Cheli et al., 1980]. This makes calibration difficult. It is therefore more beneficial to identify the potential sources of variability within the assay and to establish the specific conditions that will minimize variability for each test system and optimize the mutagenicity of that system.

The potential sources of variability within the Ames assay are quite diverse. Since this assay tests for mutant bacteria that revert from a histidine-negative (His^-) genome back to a histidine-positive (His^+) one, it is essential to monitor both the mutations that occur in the absence of a known mutagen (spontaneous revertants (SR)) and the mutations that occur in response to a known mutagen (induced revertants (IR)) [Maron and Ames, 1983; Claxton et al., 1987]. Spontaneous revertants have been classified according to when the mutations occur [Salmeen and Durisin, 1981]. The spontaneous His^+ mutant bacteria that are present in the nutrient cultures used to grow the bacteria are designated as pre-existing revertants (PER),

whereas those His+ mutations that occur after plating (i.e. during the 48-hour incubation period) are designated as plate spontaneous revertants (PSR). The number of PER is directly proportional to the initial population of bacteria per plate (n_o) [Georghiou et al,. 1989; Salmeen and Durisin, 1981]. If n_o is in the range of 10^8 bacteria per plate most of the spontaneous revertants originate from PER. If, however, n_o is 10^7 bacteria per plate, the number of spontaneous revertants decreases 10 fold, and most of the SR are PSR [Salmeen and Durisin, 1981]. Storage of permanent stock of the tester strains also affects the number of PER. In one study the ratios of PER/10^9 viable bacteria from cultures innoculated directly from differently stored permanent stock were as follows: lyophilized permanents - 267; frozen permanents - 187; master plates - 57; and single colony isolates - essentially 0 [Georghiou et al., 1989]. Several other factors have been shown to affect the number of SR. These include the (1) age of the master plates [Maron and Ames, 1983; Salmeen and Durisin 1981]; (2) type of nutrient broth used for the growth culture [Maron et al., 1981; Vithayathil et al., 1978]; (3) the amount of histidine in the top agar [deRatt et al., 1984; Maron and Ames, 1983]; (4) volume, brand, concentration, uniformity of pouring and autoclaving of bottom agar [deRatt et al., 1984; Maron and Ames, 1983; Gatehouse, 1987]; and (5) the metabolic activating system (S9 or microsomes) [Peak et al., 1982; Rossman et al., 1983; Vithayathil et al., 1978]. Acceptable ranges of spontaneous revertants for each tester strain have been determined. These

ranges are quite broad, however, so each laboratory is advised to establish its own narrower acceptable ranges [deSeries et al., 1979; Maron and Ames, 1983]. Induced revertants (IR) are much more sensitive to experimental conditions and contribute to experimental variability more so than SR [Peak et al., 1980; Grafe et al., 1981; Salmeen and Durisin, 1981]. Nutrient broth made from beef extract increases the mutagenesis of tester strains [Commoner et al., 1978; Vithayathil et al., 1978; Dolara, 1982]. The cofactor-incubation mix [Glatt et al., 1991], the metabolic activating system [Goggelmon, 1980; Peak et al., 1982, Rossman and Molina, 1983; Gatehouse and Wedd, 1984], the solvents used to dissolve the mutagens [Maron et al, 1981; Anderson and McGregor, 1980; Burnett et al, 1982] and the length of preincubation [Yahagi et al., 1975, 1977; Neudecker et al., 1989 a, b; Glatt et al., 1991] have all been shown to alter the induced mutagenicity of tester strains. Furthermore, many aspects of agar plate preparation including method of plate sterilization [Claxton et al.; 1987; Maron and Ames, 1983; Keir et al., 1986], amount of top agar used [Maron and Ames, 1983; Josephy, 1989], histidine concentration in the top agar [Maron and Ames, 1983; Aescherbacher et al., 1983; Friederich et al., 1982; Salmeen and Durisin, 1981], bottom agar volume and uniformity [Friederich et al., 1982; Cheli et al., 1980; Belser et al., 1981], and autoclaving [Friederich et al., 1982; Majeska and McGregor, 1992] affects the mutagenicity of tester strains.

In this study two *Salmonella typhimurium* strains, TA1535 and TA100, which detect mutagens that cause base pair substitutions, were used. Each strain contains a different set of mutations. These mutations have been introduced to enhance sensitivity to specific types of mutagens. Both tester strains contain the *hisG46* mutation in the hisG gene. This gene encodes the first enzyme, phosphoribosyltransferase, of the histidine biosynthetic pathway [Brenner and Ames, 1971]. This mutation prevents the bacteria from synthesizing histidine, they therefore become histidine dependent (His^-). Mutations that cause base pair substitutions revert the *hisG46* mutation back to the normal wild type and thus restore the ability of the bacteria to synthesize endogenous histidine. These revertant (His^+) bacteria survive on histidine deficient plates and produce clonal colonies which are easily counted. The deep rough mutation (*rfa*), which causes partial loss of the lipopolysaccharide barrier that coats the surface of the bacteria, is found in both strains [Ames et al., 1973]. This mutation increases the permeability of the bacteria to large molecules that have difficulty penetrating the normal cell wall. The two strains also possess the *uvrB* mutation. This mutation is a deletion of the gene that codes for DNA excision repair [Ames, 1971]. The *uvrB* gene extends through the *bio* (biotin) gene. Consequently deletion of *uvrB* function also results in deletion of *bio* gene function. These strains are unable to synthesize biotin so biotin must be included in the growth agar [Ames, 1971; Ames et al., 1973]. Mutations that hamper the tester strains' ability for DNA

repair gives more stability to the other engineered mutations that have been introduced into the host genome. Strain TA100 was derived from TA1535 by introducing the pKM101 plasmid (R-factor). This plasmid increases chemical and spontaneous mutagenesis by enhancing a normal error prone DNA repair system. It also enhances UV mutagenesis and makes TA100 sensitive to some frame shift mutagens in addition to the base-pair substitution mutagens [Langer et al., 1981; Walker and Dobson, 1979; Shanabruch and Walker, 1980]. The two strains TA1535 and TA100 were used in the following experiments.

The first set of experiments were conducted to determine the effect of the age of master plates and the age of bottom agar on mutagenicity. The plate aging effects on the mutagenicity of the tobacco specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) were compared with the plate aging effects on the mutagenicity of aflatoxin B_1 (AFB_1), benzo[a]pyrene (BaP) and N-methyl-N-nitro-N-nitrosoguanidine (MNNG). The second series of experiments were performed to determine the optimal conditions for NNK-induced mutagenesis using tester strain TA1535. In these experiments, the effects of DMSO, NNK concentration, P450 induction and microsomal concentration on NNK-induced mutagenesis were determined.

MATERIALS AND METHODS

Chemicals

4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), purity>98%, was purchased from Chemsyn Science (Lenexa, KS). Arochlor 1254 was obtained from Ultra Scientific (North Kingstown, RI). Phenobarbitol, ß-naphthoflavone, 3-methylcholanthrene, isosafrol, dexamethasone, aflatoxin B_1, benzo[a]pyrene, N-methyl-N-nitro-N-nitrosoguanidine (MNNG), ampicillin, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, $NADP^+$ and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical (St. Louis, MO). Bactoagar was purchased from VWR Scientific (Cerritos, CA) and nutrient broth No. 2 was purchased from Oxoid Co. (Basingstoke, Hants UK).

Preparation of hepatic S9 and microsome fractions

Male Sprague-Dawley rats (180-200 g, Harlen Laboratories, Indianapolis, IN), Fisher 344 rats (180-200 g, Charles River Laboratory, Wilmington, ME), and Syrian golden hamsters (150-160 g, Charles River Laboratory, Wilmington, ME) were provided water and Purina laboratory rodent chow ad libitum. The schedule for the daily i.p. administration of P450 isoenzyme inducers was as follows: phenobarbitol (PB), 60 mg/kg body weight in saline for 5 d; 3-methylcholanthrene (MC), 25 mg/kg in corn oil for 3 d; dexamethasone (DM), 100 mg/kg in saline for 4 d; isosafrol (ISF),

150 mg/kg in corn oil for 3 d; ethanol (ET), 500 mg/kg for 2 d; and ß-naphthoflavone (ß-NF), 80 mg/kg in corn oil for 5 d. This schedule gave the optimum activity from each inducer [Paolini et al., 1991]. Twenty-four hr after the last injection of inducer, the animals were sacrificed by cervical dislocation at 8:00 a.m. and rat liver S9 and hamster liver microsomes were prepared aseptically [Maron and Ames, 1983]. Animals treated with Aroclor (AR) 1254 were given a single i.p. injection (500 mg/kg) and were sacrificed five days later [Maron and Ames, 1983]. Control animals were given daily injections of saline for 5 d and sacrificed 24 hr after the last injection. Protein determinations in the S9 and microsome fractions were performed [Lowry et al., 1951] and one ml aliquots from pooled samples (3 hamsters or 2 rats) from each treatment group were frozen and maintained at -80° C (up to 6 months) until used.

Mutagenicity Assays

Incubations containing S9 or microsomal protein from a specific inducer (omitted for MNNG), mutagen (200 µl of 80 mM NNK in DMSO or saline; 0.1 µg AFB_1 in 10 µl DMSO; 2.5 µg BaP in 10 µl DMSO; 0.17 µg MNNG in 10 µl DMSO, cofactor buffer (pH 6.8) for NNK [Guttenplan, 1980]; phosphate buffer (pH 7.4) for AFB1 [O'Brien et al., 1983] and MNNG [Shirai et al., 1980]; and Tris-HCl buffer (pH 7.4) for BaP) and 0.1 ml *Salmonella typhymurium* strain TA1535 or TA100 (a gift from Dr. Bruce Ames, University of California, Berkeley) were

mixed together. For assays using TA1535 with the mutagens NNK and MNNG, the mixtures were preincubated in a shaking waterbath for 30 minutes at 37° C [Yahagi et al., 1975; 1977]. Two ml top agar was then added to each sample and the contents were poured onto minimal essential agar plates [Maron and Ames, 1983]. The top agar was allowed to solidify and the plates were then incubated at 37° C for 48 hr. The induced histidine-independent (His^+) revertant colonies were then scored and the spontaneous revertant colonies were subtracted. The results were then expressed as the mean number of induced revertant colonies/plate.

The master plates were prepared from frozen permanent samples [Maron and Ames, 1983]. They were then placed in plastic bags, sealed and stored at 4° C until needed. The bottom agar plates were prepared as described [Maron and Ames, 1983] and stored at 4° C until needed. Both the master plates and the agar plates were prepared in advance and stored for the requisite time period so that each set of experiments could be performed concurrently (i.e. same day, same bacteria, same S9, same media, same laboratory, etc.) [Claxton et al., 1991c; Myers et al., 1987].

Statistical analysis

Data were analyzed by the student's unpaired t-test using the MacIntosh Stat-view (version 1.04) software. Statistical significance was defined as $p \leq 0.05$ compared to control values [Myers et al., 1987; Seiler et al., 1980].

RESULTS

Age of Master Plate

The effect of the age of master plates on mutagenesis is depicted in Figures 1 and 2. Nutrient broth cultures of *Salmonella typhimurium* strains TA1535 and TA100 were grown from master plates that were 0, 1, 2, 3, or 4 wks old. These cultures were then used with Arochlor-induced Fisher 344 rat liver S9 and the following mutagens: NNK and MNNG for TA1535; AFB_1, BaP and MNNG for TA100. NNK-induced about one half the revertants in cultures prepared from 4 wk old master plates compared with the induced revertants from cultures from fresh (0 wk) master plates (Figure 1A). Age of the master plate had a more pronounced effect on MNNG-induced mutagenesis in TA1535. With cultures grown from 3 wk old master plates, the MNNG-induced revertants (IR) decreased to about the spontaneous revertant (SR) level (Figure 1B). BaP and AFB_1 were not mutagenic to TA1535 in our studies as previously reported [Wheeler et al., 1981; deFlora et al., 1984]. The MNNG-induced mutagenicity in TA1535 has been shown to be enhanced by using phosphate buffer (pH 7.4) and a 30 min preincubation [Shirai et al., 1980; Balansky and Bryson, 1985]. The spontaneous revertants of TA1535 were unaffected by master plate age in our studies. There was no significant variability of AFB_1 and BaP-induced mutagenicity in TA100 cultures relative to age of master plates (Figure 2A,B). There was, however, a significant

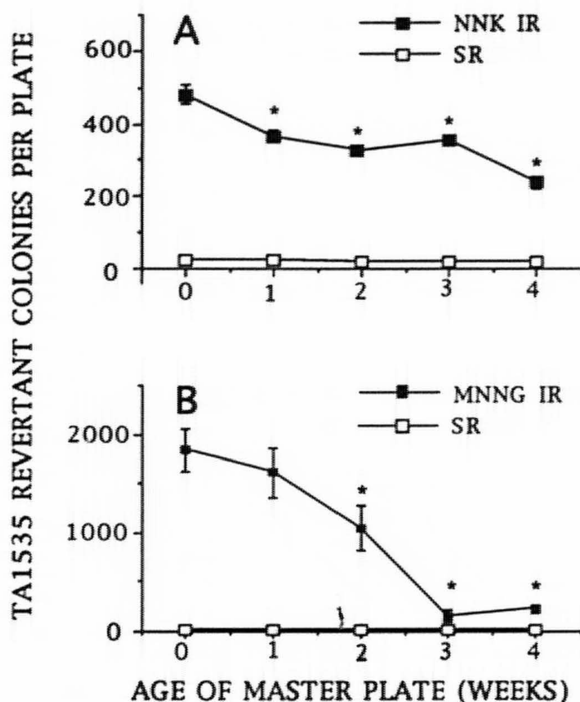
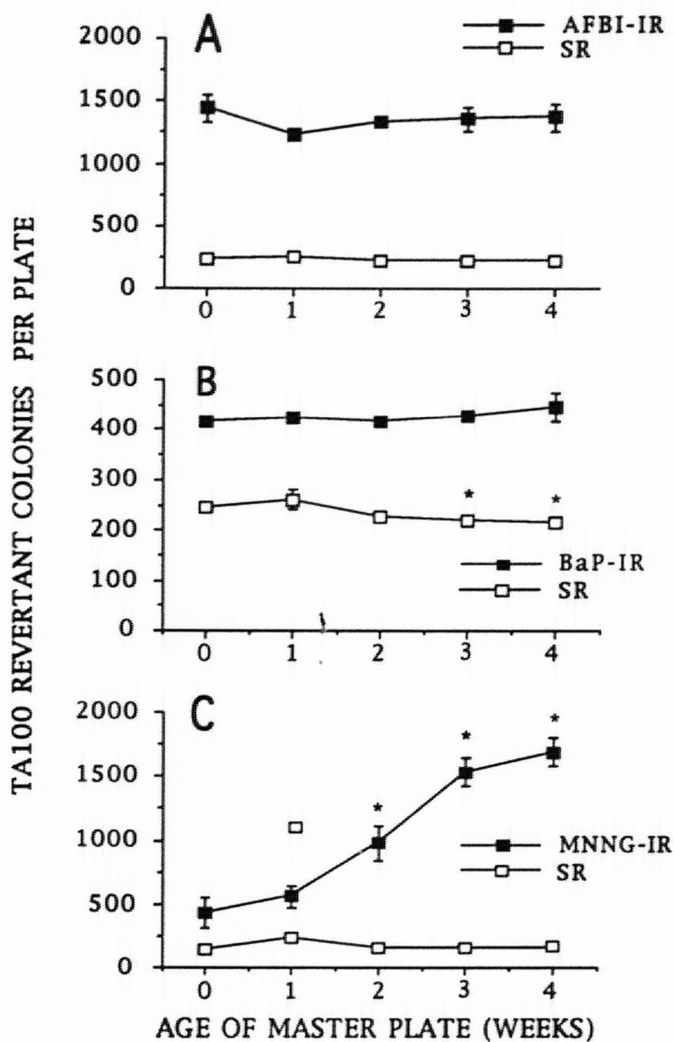


Figure 1: The effect of master plate age on mutagenesis in *Salmonella typhimurium* TA1535 using Arochlor induced Fisher 344 rat liver S9. (A) NNK-induced revertant colonies (NNK-IR); (B) MNNG-induced revertant colonies (MNNG-IR). Spontaneous revertants (SR) are shown. Data points represent the means (N=3) and bar lines represent +/- SEM. Where bar lines are not shown the errors fall within the area of the symbol. Statistically significant ($p < 0.05$) differences from controls are indicated with an asterisk *.

Figure 2: The effect of master plate age on mutagenesis in *Salmonella typhimurium* TA100 using Arochlor induced Fisher 344 rat liver S9. (A) AFB_1-induced revertant colonies (AFB_1-IR); (B) BaP-induced revertant colonies (BaP-IR); (C) MNNG-induced revertant colonies (MNNG-IR). Spontaneous revertant colonies (SR) are shown. Data points represent the means (N=3) and bar lines represent ± SEM. Where bar lines are not shown the errors fall within the area of the symbol. Statistically significant ($p < 0.05$) differences from controls are indicated with an asterisk *.


A
AFBI-IR
SR
2000
1500
1000
500
0
0
1
2
3
4
B
500
400
300
200
100
0
*
*
BaP-IR
SR
0
1
2
3
4
C
2000
1500
1000
500
0
*
*
*
MNNG-IR
SR
0
1
2
3
4
TA100 REVERTANT COLONIES PER PLATE
AGE OF MASTER PLATE (WEEKS)

($p \leq 0.05$) increase in MNNG-induced mutagenicity (Figure 2C) with age of master plate. The MNNG-induced revertant colonies in cultures from 4 wk-old master plates were about 3-fold greater than the IR from cultures from fresh (0 wk) master plates. The SR in TA100 remained relatively constant. These experiments were performed using plates with freshly prepared bottom agar.

Age of bottom agar

The effect of age of bottom agar is shown in Figures 3 and 4. Minimal essential medium agar plates were prepared and then stored at 4° C for up to 4 wks. Nutrient broth cultures of TA1535 and TA100 were prepared from fresh master plates. The mutagenicity of NNK and MNNG with TA1535; and AFB_1, BaP and MNNG with TA100 was determined using Arochlor-induced Sprague-Dawley rat liver S9 and agar plates of various ages. NNK-induced mutagenicity was enhanced about 3-fold between the fresh agar plates (wk 0) and the 4 wk old agar plates (Figure 3A). Age of agar plates had the opposite effect on MNNG-induced revertants in TA1535. The number of SR was relatively constant irrespective of the age of agar plate. As agar plates aged, there was an enhanced effect on the mutagenicity of AFB_1, BaP, and MNNG in TA100 (Figure 4) and was most pronounced with 4 wk old plates. MNNG-induced mutagenicity in TA100 was the most sensitive to age of agar plates (Figure 4C). These experiments were performed as concurrently as possible.

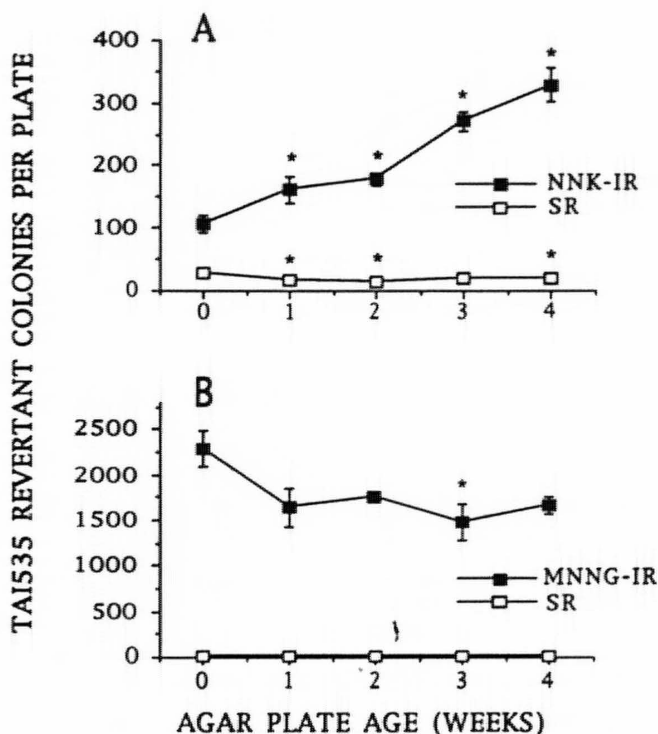
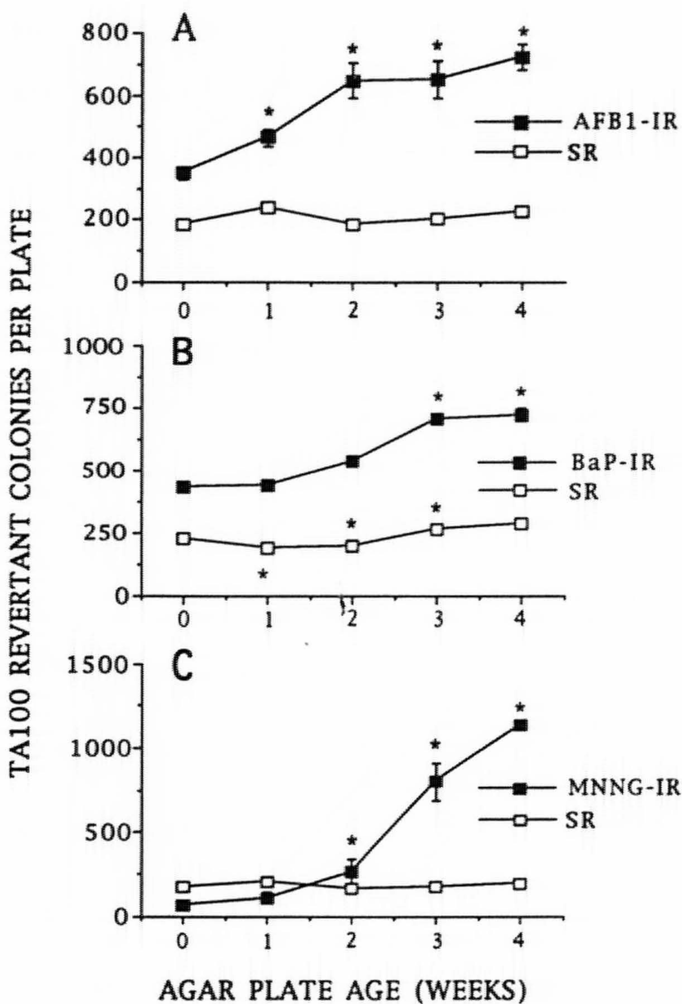


Figure 3: The effect of agar plate age on mutagenesis in *Salmonella typhimurium* TA1535 using Arochlor induced Sprague-Dawley rat liver S9. (A) NNK-induced revertant colonies (NNK-IR); (B) MNNG-induced revertant colonies (MNNG-IR). Spontaneous revertants (SR) are shown. Data points represent the means (N=3) and bar lines represent +/- SEM. Where bar lines are not shown the errors fall within the area of the symbol. Statistically significant ($p < 0.05$) differences from controls are indicated with an asterisk *.

Figure 4: The effect of master plate age on mutagenesis in *Salmonella typhimurium* TA100 using Arochlor induced Sprague-Dawley rat liver S9. (A) AFB_1-induced revertant colonies (AFB_1-IR); (B) BaP-induced revertant colonies (BaP-IR); (C) MNNG-induced revertant colonies (MNNG-IR). Spontaneous revertant colonies (SR) are shown. Data points represent the means (N=3) and bar lines represent ± SEM. Where bar lines are not shown the errors fall within the area of the symbol. Statistically significant ($p < 0.05$) differences from controls are indicated with an asterisk *.


A
B
C
800
600
400
200
0
1000
750
500
250
1500
0
1
2
3
4
AFB1-IR
SR
BaP-IR
MNNG-IR
TA100 REVERTANT COLONIES PER PLATE
AGAR PLATE AGE (WEEKS)

Effect of DMSO on NNK-induced Mutagenicity in TA1535

The effects of different cytochrome P450 inducers on Fisher 344 rat liver S9 mediated mutagenicity in *Salmonella typhimurium* TA1535 is shown in Figure 5. DMSO (200 µl), solvent for NNK, inhibited the mutagenicity of NNK by S9 compared with an equal vol of saline as solvent irrespective of the cytochrome P450 induction. The relative effectiveness of the different induced S9 metabolic activating systems for mediating NNK-induced mutagenesis with NNK dissolved in saline was as follows: AR (389 ± 64.83) > PB (255 ± 11.59) > ISF (224 ± 4.00) > DM (146 ± 9.00) > MC (111 ± 10.93) > ET (34 ± 5.04). S9 from ethanol (ET) induced animals produced the fewest number of revertant colonies.

Dose reponse experiments were conducted to determine the minimum volume of DMSO that could be used without affecting NNK-induced mutagenesis in TA1535 (Figures 6 and 7). Induced Fisher 344 rat liver S9 was used for metabolic activation of NNK. All P450 inductions were sensitive to 10 µl DMSO except for S9 from MC-induced animals. Significant inhibition of NNK-induced mutagenesis by MC-induced S9 occurred at 50 µl of DMSO (Figure 6B). At the maximum dose of 200 µl DMSO, mutagenicity was reduced to about one-half the His^+ revertant colonies/plate for all the S9 inductions except DM. DM-induced S9 was most sensitive to DMSO (Figure 7B).

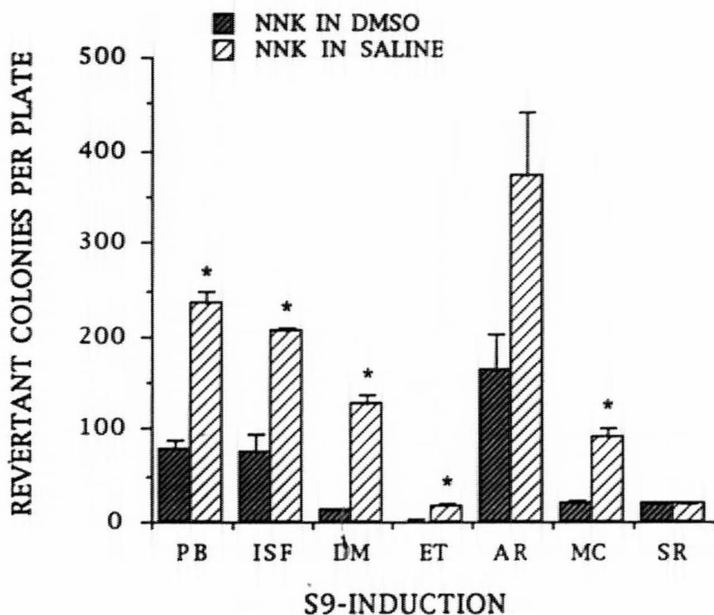


Figure 5: Comparison of solvent on NNK-induced mutagenesis in *Salmonella typhimurium* TA1535 using induced Fisher 344 rat liver S9. Data points represent the means (N=3) and bar lines represent +/- SEM. Where bar lines are not shown the errors fall within the area of the symbol. Stastistically significant ($p < 0.05$) differences from controls are indicated with an asterisk *.

Figure 6: The effect of DMSO on NNK-induced mutagenesis in *Salmonella typhimurium* TA1535 using induced Fisher 344 rat liver S9. (A) Isosafrol (ISF)-induced S9; (B) Methylcholanthrene (MC)-induced S9; (C) Phenobarbitol (PB)-induced S9. Data points represent the means (N=3) and bar lines represent ± SEM. Where bar lines are not shown the errors fall within the area of the symbol. Statistically significant ($p < 0.05$) differences from controls are indicated with an asterisk *.

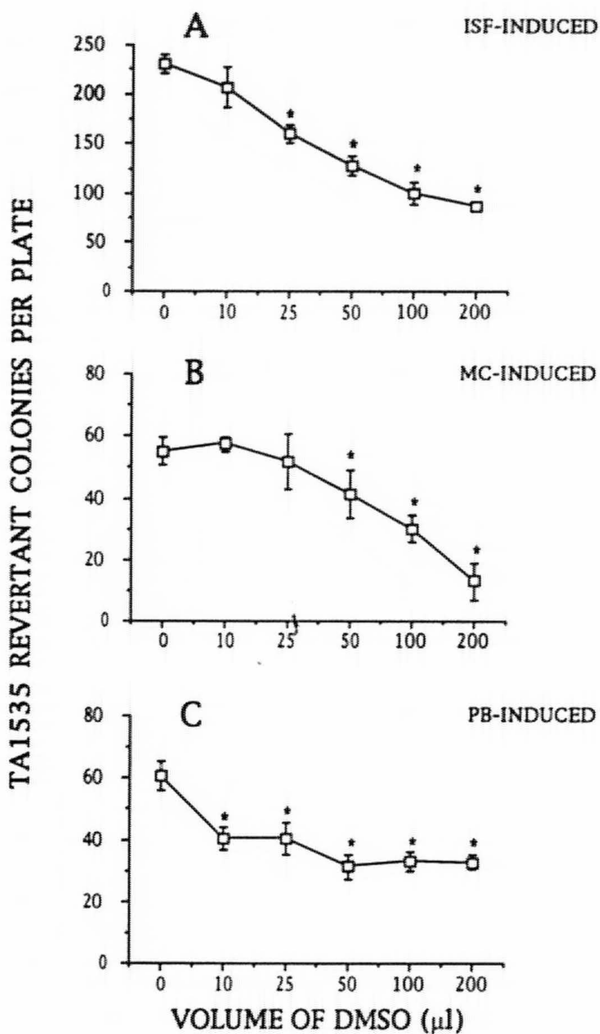
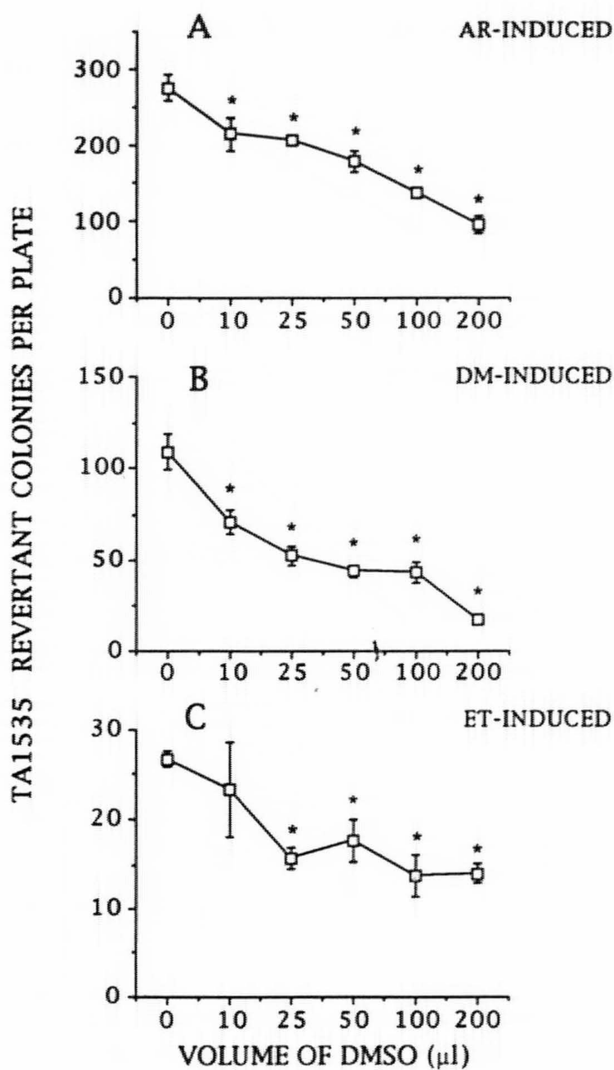

A
ISF-INDUCED
250
200
150
100
50
0
0
10
25
50
100
200
B
MC-INDUCED
80
60
40
20
0
0
10
25
50
100
200
C
PB-INDUCED
80
60
40
20
0
0
10
25
50
100
200
TA1535 REVERTANT COLONIES PER PLATE
VOLUME OF DMSO (µl)

Figure 7: The effect of DMSO on NNK-induced mutagenesis in *Salmonella typhimurium* TA1535 using induced Fisher 344 rat liver S9. (A) Arochlor (AR)-induced S9; (B) Dexamethasone (DM)- induced S9; (C) Ethanol (ET)-induced S9. Data points represent the means (N=3) and bar lines represent ± SEM. Where bar lines are not shown the errors fall within the area of the symbol. Statistically significant ($p < 0.05$) differences from controls are indicated with an asterisk *.



A
AR-INDUCED
300
200
100
0
0 10 25 50 100 200
B
DM-INDUCED
150
100
50
0
0 10 25 50 100 200
C
ET-INDUCED
30
20
10
0
0 10 25 50 100 200
TA1535 REVERTANT COLONIES PER PLATE
VOLUME OF DMSO (μl)

To determine whether the observed effects of DMSO were due to a decreased number of viable bacteria, a DMSO toxicity test was done. Toxicity to DMSO in TA1535 occurred with volumes greater than 100 µl (Figure 8).

Optimizing NNK Mutagenesis

Dose response curves for NNK were performed to determine the optimal concentration of NNK in saline for induced mutagenesis in TA1535 (Figures 9 and 10). Induced Fisher 344 rat liver S9 was used for metabolic activation. All P450 inductions mediated fairly linear dose responses to NNK-induced mutagenesis. There was no evidence of NNK-induced toxicity at the highest concentration of NNK (80 mM).

Figure 11 shows the results of the concentration of hamster liver microsome protein on NNK-induced mutagenesis in TA1535. These experiments were done using 80 mM NNK in saline (100 µl) in the incubation mixtures. Maximum mutagenesis mediated by non-induced and ß-NF induced microsomes occurred at 1.0 mg protein/plate (Figure 11A,C) whereas maximum mutagenesis mediated by PB-induced microsomes occurred at 1.5 mg protein/plate (Figure 11B). The number of revertant colonies induced by NNK using 1.0 mg hamster liver microsome protein was 143 ± 8.19 for noninduced, 166 ± 15.96 for PB-induced and 271 ± 13.97 for ß-NF-induced.

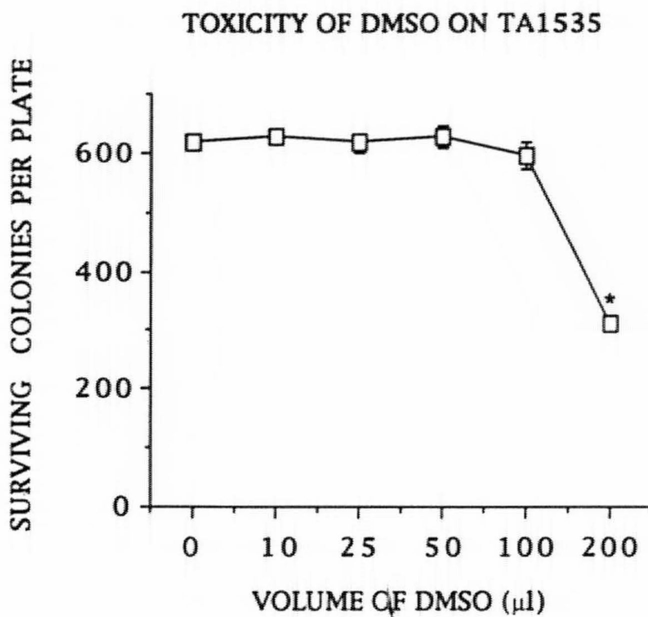


Figure 8: Toxic effects of DMSO on *Salmonella typhimurium* TA1535 as indicated by the number or surviving colonies on histidine supplemented plates. Data points represent the means (N=3) and bar lines represent +/- SEM. Where bar lines are not shown the errors fall within the area of the symbol. Statistically significant ($p < 0.05$) differences from controls (0 µl DMSO) are indicated with an asterisk *.

Figure 9: Dose response of NNK-induced mutagenesis in *Salmonella typhimurium* TA1535 using induced Fisher 344 rat liver S9. (A) Isosafrol (ISF)-induced S9; (B) Methylcholanthrene (MC)-induced S9; (C) Phenobarbitol (PB)-induced S9. Data points represent the means (N=3) and bar lines represent +/- SEM. Where bar lines are not shown the errors fall within the area of the symbol. Statistically significant ($p < 0.05$) differences from controls are indicated with an asterisk *.

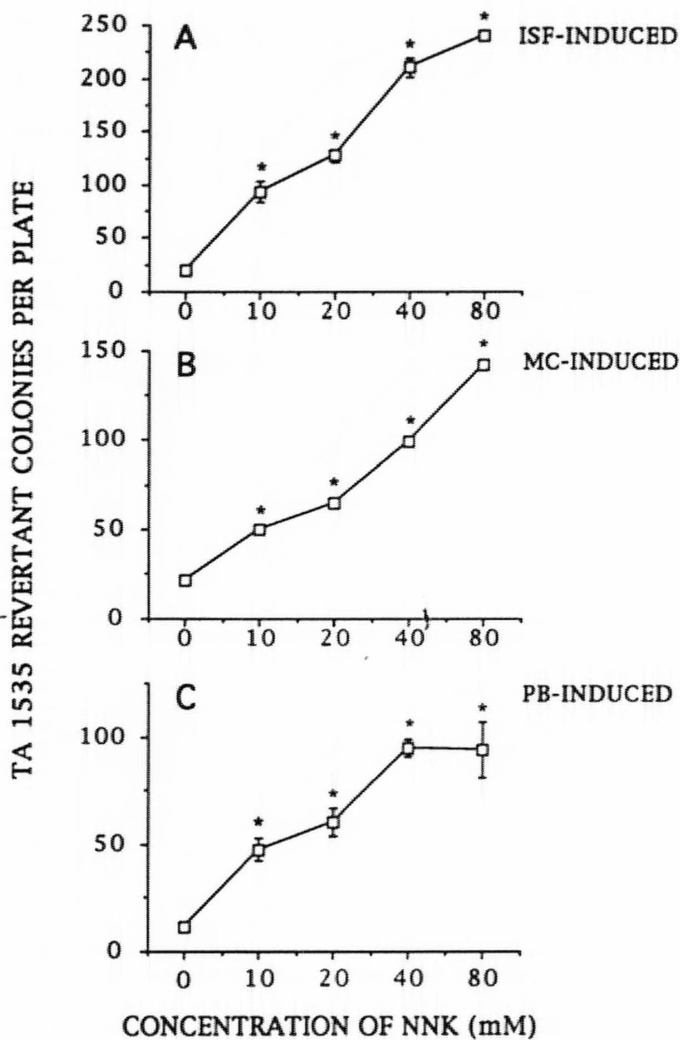

TA 1535 REVERTANT COLONIES PER PLATE
A
ISF-INDUCED
250
200
150
100
50
0
0
10
20
40
80
B
MC-INDUCED
150
100
50
0
0
10
20
40
80
C
PB-INDUCED
100
50
0
0
10
20
40
80
CONCENTRATION OF NNK (mM)

Figure 10: Effect of concentration of NNK on mutagenesis in *Salmonella typhimurium* TA1535 using induced Fisher 344 rat liver S9. (A) Arochlor (AR)-induced S9; (B) Dexamethasone (DM)-induced S9; (C) ß-naphthaflavone (ß-NF)-induced S9. Data points represent the means (N=3) and bar lines represent ± SEM. Where bar lines are not shown the errors fall within the area of the symbol. Statistically significant ($p < 0.05$) differences from controls are indicated with an asterisk *.

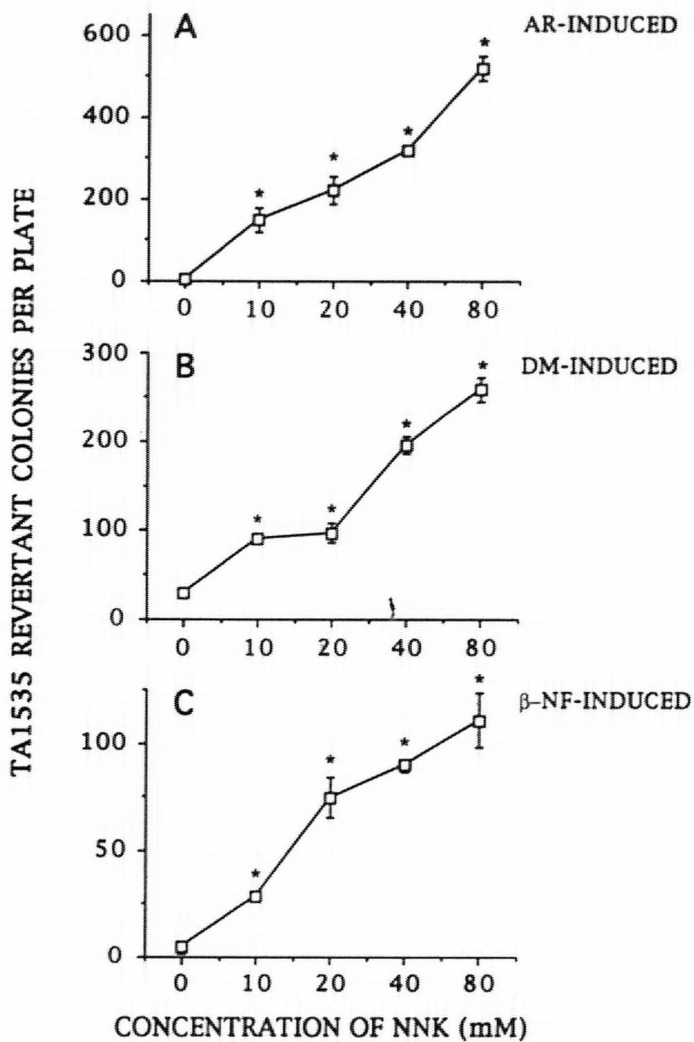
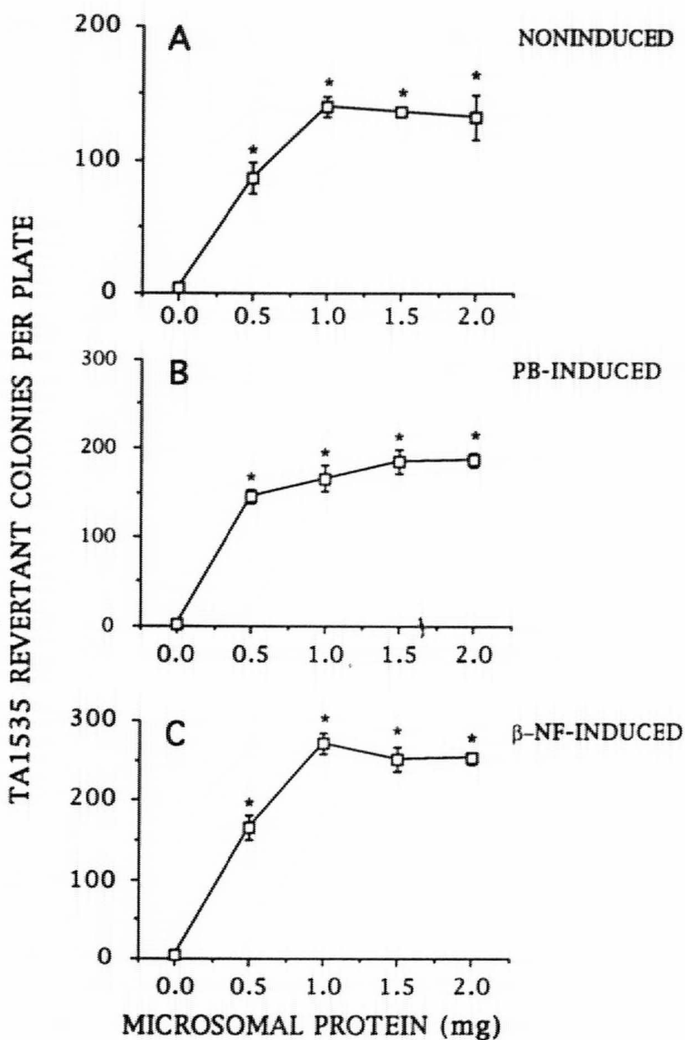

TA1535 REVERTANT COLONIES PER PLATE
A
AR-INDUCED
600
400
200
0
0
10
20
40
80
B
DM-INDUCED
300
200
100
0
0
10
20
40
80
C
β–NF-INDUCED
100
50
0
0
10
20
40
80
CONCENTRATION OF NNK (mM)

Figure 11: Effect of concentration of hamster liver microsome protein on NNK-induced mutagenesis in *Salmonella typhimurium* TA1535. (A) Noninduced microsomes (NI); (B) Phenobarbitol (PB)-induced microsomes; (C) ß-naphthaflavone (ß-NF)-induced microsomes. Data points represent the means (N=3) and bar lines represent ± SEM. Where bar lines are not shown the errors fall within the area of the symbol. Statistically significant ($p < 0.05$) differences from controls are indicated with an asterisk *.


A
NONINDUCED
B
PB-INDUCED
C
β-NF-INDUCED
TA1535 REVERTANT COLONIES PER PLATE
MICROSOMAL PROTEIN (mg)
0.0
0.5
1.0
1.5
2.0
0
100
200
300

DISCUSSION

Plate Aging Effects

The Salmonella/mammalian microsome assay is a useful tool for determining the mutagenicity of many chemicals and complex mixtures. Mutagenicity studies are important because of the high correlation between mutagenicity and carcinogenesis. Eighty to ninety percent of substances that are mutagenic are also carcinogenic [Bartsch et al., 1980; Ames and McCann, 1981; Maron and Ames, 1983; Ashby and Tennant, 1988]. Extensive work has been done to make this assay as reliable as possible [Claxton et al., 1991 b, c; Belser et al., 1981; Grafe et al., 1981; Stead et al., 1981]. A good indicator of reliability is whether the number of spontaneous revertant colonies/plate (SR) and the number of induced revertant colonies/plate (IR) remain relatively constant in repeated experiments [Claxton et al., 1987; Gatehouse, 1987; Myers et al., 1987]. Experimental variability can be minimized if the causes of variability are identified and guidelines are established to enhance reproducibility. General guidelines have been published [Belser et al., 1981; deSerres and Shelby, 1979; Cheli et al., 1980]. It is imperative, however, that for each mutagen, specific experimental conditions be established to optimize that mutagenic system .

To optimize conditions for studies of NNK-induced mutagenesis in *Salmonella typhimurium* TA1535, we examined the effects of age of master plate and age of bottom agar on the mutagenesis of several

mutagens including AFB_1, BaP, NNK and MNNG. When investigating Salmonella mutagenesis, availability of a convenient stock of bacteria is essential. Maron and Ames [1983] recommend several methods for storing the bacterial strains including the use of master plates. Nutrient broth cultures grown from master plates contain fewer pre-existing revertant colonies (PER) compared to cultures grown from other stored permanents [Georghiou et al., 1989]. Master plates are themselves a potential source of experimental variability. Although Maron and Ames [1983] indicate that master plates stored at 4° C are stable for two months, our studies showed that mutagenesis in Salmonella tester strains was affected by age of master plate. Figure 1A shows that NNK-induced mutagenicity decreased in a dose-dependent manner. Cultures from 4 wk old master plates produced only half the NNK-induced revertants compared to cultures from newly prepared (0 wk) master plates. Response of TA1535 to MNNG was similar to the response to NNK (Figure 1B). With the tester strain TA100, MNNG-induced mutagenicity was enhanced in cultures from older master plates. AFB_1 and BaP-induced revertants were unaffected by age of master plate. Maron and Ames [1983] argue that an increase in SR is a good indicator of master plate aging. Our studies show that as the TA1535 master plate ages, the SR remained relatively constant even though there were statistically significant ($p \leq 0.05$) decreases in the IR by NNK and MNNG. The SR in our studies was not a reliable indicator of master plate age. Saleem and Durisin [1981] showed that SR do not change significantly

when comparing cultures from 10 and 21 day old master plates which supports our findings. The BaP control (SR) was the only control tested that showed a significant increase in SR with age of master plate. Cultures from 3 and 4 wk-old master plates produced a small but significantly ($p \leq 0.05$) fewer number of SR than cultures from younger master plates. These SR are false positive indicators since there was no corresponding change in the number of BaP-induced revertants (Figure 1B).

Several conclusions can be drawn from these results. Firstly, aging of master plates does not affect all tester strains alike. With TA1535, aged master plates produced cultures that were less sensitive to the nitrosamine mutagens NNK and MNNG. This aging effect does not appear to be due to changes in the metabolism of the mutagen because both the indirect acting (NNK) and the direct acting (MNNG) nitrosamine produced fewer IR in cultures from aged master plates. Salmonella strain TA100, on the other hand, was resistant (at least for AFB1 and BaP) to master plate aging during the 4 wks tested. A second conclusion is that the response to a particular mutagen with master plate aging is not always consistent between tester strains. With strain TA1535, cultures from aged master plates exhibited a decrease in IR in response to MNNG whereas cultures from aging master plates of TA100 exhibited an increase in MNNG IR. Another conclusion is that SR are poor indicators of master plate aging. With NNK and MNNG the SR were false negatives and for BaP there were false positives. Rather than relying on SR as an indicator

of master plate age, positive controls should be run for each experiment. A final conclusion is that all mutagens are not equally sensitive to master plate aging. There were statistically significant changes in the number of IR with NNK and MNNG with cultures from master plates that had aged as little as one wk, whereas BaP and AFB_1 IR were unaffected by master plate age for the 4 wks tested. An awareness of how specific mutagen systems are affected by master plate age allows one to established guidelines for the preparation of master plates. For studies of NNK-induced mutagenesis, master plates should not be older than one week.

The effect of the amount, type (brand), and composition of agar on mutagenesis has been studied extensively [deRaat et al., 1984; Friederich et al., 1982; Belser et al., 1981; Seiler et al., 1980]. Mode of preparation of agar plates can also affect mutagenesis. Several studies have shown that when glucose is autoclaved together with the Vogel Bonner salts [1956] there is enhanced mutagenicity [Friederich et al., 1982; Majeska and McGregor, 1990; 1992]. We observed a similar effect with the response of TA100 to BaP, AFB_1, and MNNG. Autoclaving glucose with the Vogel Bonner salts significantly ($p \leq 0.05$) increased mutagenicity of BaP, AFB_1 and MNNG by 12.57%, 16.84% and 35.74% respectively (data not shown). In tester strain TA1535, in response to NNK, there was an opposite effect. NNK mutagenicity was significantly ($p \leq 0.05$) lower (23.46%) when using plates with all the agar components autoclaved together. TA1535 response to MNNG showed the same trend as to NNK

although the decrease in mutagenicity with autoclaved glucose was not statistically significant (data not shown).

The age of bottom agar may also influence mutagenesis. Maron and Ames [1983] indicated that agar plates can be stored for up to two months if wrapped in plastic bags and kept at 4° C. In a short term study (8 d) done by Friederich et al. [1982] the storage of agar plates at 4° C and at room temperature (25° C) was compared. The method of storage and age of the plates showed no differences in the number of SR or IR. They recommended that the plates be stored at room temperature for 2-3 d before transferring them to 4° C to reduce condensation on the lids. Our studies showed that NNK-induced mutagenesis in TA1535 increased significantly ($p \leq 0.05$), in a dose response manner, with age of bottom agar. This effect began with plates that were only one wk old and continued for the entire period of 4 wks (Figure 3A). The response of TA100 to AFB1, BaP and MNNG was similar although the enhanced mutagenicity to MNNG was not statistically significant until wk 2 and not to BaP until wk 3 (Figure 4). MNNG-induced mutagenesis in TA1535 was opposite to the response of TA100 to age of bottom agar. IR in response to MNNG decreased significantly with agar plates that were 3 wks old (Figure 3B).

There are several plausible explanations for these observed changes in mutagenicity. With time, evaporation occurs, even when plates are stored at 4° C. This results in a decrease in volume of bottom agar, expressed both as an increase and a decrease in IR

[Belser et al., 1981; Friederich et al., 1982; Seiler et al., 1980]. The expression of SR is also affected by agar volume. In one study using TA100 it was demonstrated that with 30 ml agar, 200 SR/plate were produced whereas with 15 ml agar there were less than 100 SR/plate. Within the volume range of 15-30 ml, each 5 ml difference in agar volume corresponded to a difference of 20-30 SR [deRaat et al., 1984]. Evaporative loss of water from the agar could also result in increased concentrations of the agar components. Increasing the percent of agar, while keeping the volume constant, increased mutagenicity in TA1535 [deRaat et al., 1984]. Other studies show that alteration of the concentration of the Vogel Bonner salts affects mutagenicity. Decreasing the Vogel Bonner salt concentration in the agar resulted in a decrease in the mutagenicity of sodium azide in TA1535 and TA100 [Witmer et al., 1982]. Agar also contains endogenous mutagens and toxins, the concentration and/or potency of which can be increased or decreased depending in part on how the agar is prepared and how rapidly it is cooled [deRaat et al., 1984; Majeska and McGregor, 1992; Witmer et al., 1982]. Dessication and aging of agar may increase the concentration of endogenous mutagens and toxic substances. All of these factors may contribute to the changes in mutagenicity that occur as bottom agar ages, although each mutagenic system may be affected differently.

The difference in the effects of MNNG-induced mutagenicity we observed in TA100 and TA1535 deserves explanation. Cheli et al. [1980] showed that even with carefully controlled protocols and

repeated experiments, experimental variability still occurs. Each tester strain appears to have its own intrinsic variability. The R-factor strains (TA100 and TA98) appear to be less variable than the corresponding non-plasmid strains (TA1535 and TA1538) respectively, even though the non-plasmid strains have fewer spontaneous revertants. Mutagens also express inherent variability. When comparing MNNG-IR between strains, there was greater variability with TA1535 than with the TA100 tester strain [Cheli et al., 1980]. Variability is also evident when examining MNNG mutagenic dose response curves [Claxton et al., 1991b]. Most mutagens have linear dose response curves but for some mutagens, like MNNG, the mutagenic dose response curves are non-linear [Maron and Ames, 1983; McCann et al., 1975]. Tester strains have been shown to be most sensitive to mutagens when they are in logarithmic growth phase. The growth curves of TA100 and TA1535 revertant colonies in agar indicate that the His+ revertants are in log phase growth at 6 and 4 hrs respectively [Barber et al., 1983]. This would imply that the bacteria are most sensitive to mutagens 4 to 5 hrs after plating. The half life of MNNG is about 2 hrs on agar plates [Awerbach and Sinskey, 1980]. A mismatch occurs in this system between the time the bacteria are sensitive to the mutagen and when the MNNG is most active. To overcome this mismatch, Guttenplan [1980] has shown that the half life of MNNG can be extended in an acidic medium with maximum stability occurring at pH 5.5. Barber et al. [1983] demonstrated that the addition of MNNG

6-8 hrs after plating the bacteria enhanced the mutagenicity of MNNG. These results indicate that there exists an interdependence of the growth of bacteria on the plates and the reversion of these bacteria. We were initially unaware of this interdependence. In our experiments, it appears that the reversion curves and the tester strain growth curves were out of phase with each other. Coupled with the intrinsic variability of MNNG especially with tester strain TA1535, it appears that MNNG may not have been an appropriate mutagen to use. Because of the complex kinetics involved in MNNG-mutagenesis and its inherent variability, our studies indicate that caution should be exercised in making any generalizations from our studies based on the mutagenic activity of MNNG.

Optimizing Conditions for NNK Mutagenesis

4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), a nicotine derivative, is a potent carcinogen which is implicated in the causation of oral cancers in tobacco chewers and lung cancers in smokers [Hecht and Hoffmann, 1988]. NNK induces tumors in the lung, nasal mucosa, trachea, liver and pancreas of rodents [Castonguay et al., 1991]. Cytochrome P450 enzymes play an important role in the activation of most carcinogens [Guengerich, 1991; Conney, 1982]. NNK also requires metabolic activation to form reactive products that bind to DNA [Hecht and Hoffmann, 1988]. The cytochromes P450 2E1, 1A2, 2A3, 2B1, 2D6 are all involved in NNK metabolism [Crespi et al., 1991; Guo et al., 1991]. Novel forms of

cytochrome P450 can be induced by the administration of specific chemicals [Okey, 1990; Paolini et al., 1991]. The two major inducible isoenzymes from phenobarbitol (PB) treatment are P450 2B1 and P450 2B2 [Adesnik and Atchison, 1985]. Methyl- cholanthrene (MC) and ß-naphthoflavone (ß-NF) induces P450 1A1 and P450 1A2 [Nebert and Gonzalez, 1987]. Isosafrol (ISF) induction produces both PB-type and MC-type P450 isoenzymes [Okey, 1990]. Dexamethasone (DM) induces the 3A family of P450 isoenzymes [Emi et al., 1990] and ethanol (ET) induces P450 2E1 [Yoo et al., 1990]. Aroclor 1254 (AR) is a broad spectrum inducer of P450 isoenzymes [Safe et al., 1985].

Our results show that some induction procedures are better metabolic activators for NNK-induced mutagenicity with *Salmonella typhimurium* TA1535 than others (Figure 5). Aroclor-induced Fisher rat liver S9 was the best metabolic activator of NNK-induced mutagenesis. This is expected since AR is a general broad spectrum inducer. PB induction was superior to ISF although ISF induces both MC-type P450 and PB-type P450. The PB-inducible isoenzymes of cytochrome P450 (2B1 and 2B2) appear to be more active NNK metabolizers than the MC-induced 1A1 and 1A2. Non induced and ß-NF-induced S9 produced about the same number of revertants as PB-induced S9 (data not shown). Induced hamster S9 displays a different mutagenic profile than the rat: AR = ISF > PB > MC > DM > ET [Teel, 1992]. In addition, induced hamster S9 presents a different mutagenic profile than induced microsomes from the same animals.

DMSO is a commonly suggested solvent for chemicals that are otherwise difficult to put into solution [Maron et al., 1981; Anderson and McGregor, 1980]. Our protocol initially called for the use of DMSO as a solvent for NNK. A comparative effect of DMSO and the mutagenicity of NNK in the presence of induced S9 from rats indicated that DMSO significantly inhibited mutagenesis (Figure 5) irrespective of the induction system. The volume of DMSO used was 200 µl. The inhibitory effect was dose-dependent in each S9 induction system (Figures 6 and 7). A study of the effect of the volume of DMSO on survival of TA1535 (Figure 8) indicated that DMSO was toxic at volumes between 100 and 200 µl. The effect on S9-mediated mutagenesis of NNK at smaller volumes of DMSO (Figures 6 and 7) supports an inhibition of S9 metabolic activity as recently reported [Teel, 1992]. S9 from PB-induced rats was quite sensitive to 10 µl DMSO. The effects of DMSO shown in Figure 5 are most likely effects of toxicity to TA1535 and not an inhibition of the S9-mediated mutagenicity of NNK by DMSO as suggested in Figures 6 and 7. These results prompted us to minimize the use of DMSO in our experiments and to keep the volume of DMSO constant in all samples in a particular experiment. Saline was used as the solvent for NNK in subsequent studies.

To determine the appropriate concentration of NNK to use in later studies of mutagenesis, we followed the procedure of Claxton et al. [1991a]. Using a constant amount of liver S9 protein (1.74 mg/plate), we varied the concentration of NNK. The NNK dose response curves

were fairly linear (Figures 9 and 10). Maron and Ames [1983] recommended that the concentration of a mutagen used should fall within the linear portion of the dose response curve. To maximize the sensitivity of the assay, a concentration of mutagen near the maximum effect should be used [Claxton et al, 1991a]. We did not test concentrations of NNK greater than 80 mM and there was no evidence of NNK-induced toxicity at 80 mM.

Previous studies indicate that the number of IR increases as the concentration of S9 or microsome protein is increased up to a maximum. This is followed by a plateau or a decrease in the number of IR [Foster et al., 1980 a,b]. Using a concentration of mutagen that approximates the maximum number of IR, one can optimize the amount of S9 or microsome protein [Claxton et al., 1991a]. The decision to use 1 mg of hamster liver protein to activate NNK (80 mM) was based upon data presented in Figure 11. In studies described in chapters 3-5 that relate to NNK-induced mutagenesis, we utilized 80 mM NNK and 1 mg of noninduced, PB-induced or ß-NF-induced hamster liver microsomes. The use of hamster liver microsomes in place of rat liver S9 was based upon observations that hamster liver microsomes are much more efficient metabolizers of NNK [Zhang et al., 1993].

The Ames assay is a useful method of determining the mutagenicity of chemicals. The assay has identified over 1500 pure chemicals as mutagens [Claxton et al., 1988]. The *Salmonella typhimurium* tester strains have been genetically engineered to

express maximal sensitivity to different classes of mutagens. Their altered DNA repair systems limit their ability to correct the induced mutations. The cell wall of the tester strains is modified to facilitate diffusion of large complex chemicals into the cells [Maron and Ames, 1983]. These "engineered" alterations make the tester strains extremely sensitive to mutagenic agents, and unfortunately, also to experimental variability [Speck and Rosenkranz, 1975; McCoy et al., 1979]. Since tester strains differ and mutagenic effects differ, it is important to optimize conditions for enhancing sensitivity and minimizing variability for each test system. The results of studies presented in this chapter indicate that the optimal conditions for NNK-induced mutagenicity in *Salmonella typhimurium* strain TA1535 in our laboratory are: concurrent testing, using master plates and agar plates no older than 1 wk, using 1 mg hamster liver microsomal protein/plate and minimizing the volume of DMSO (≤ 10 µl) used as solvent.

REFERENCES

Adesnik M, Atchison M (1985): Genes for cytochrome P-450 and their regulation. CRC Crit Rev Biochem 19:247-305.

Aeschbacher HU, Friederich U, Seiler JP (1983): Criteria for the standardization of Salmonella mutagenicity tests: results of a collaborative study. III. The influence of the composition and preparation of the minimal medium in the Salmonella mutagenicity test. Teratogenesis Carcinog Mutagen 3:195-203.

Ames BN (1971): The detection of chemical mutagens with enteric bacteria. In: A Hollaenden (Ed.) "Chemical mutagens, principles and methods for their detection." New York, Plenum, 1:267-282.

Ames BN, Lee FD, Durston WE (1973): An improved bacterial test system for the detection and classification of mutagens and carcinogens. Proc Natl Acad Sci (USA) 70:782-786.

Ames BN, McCann J (1981): Validation of the Salmonella test: A reply to Rinkus and Legator. Cancer Res 41:4192-4196.

Ames BN, McCann J, Yamasaki E (1975): Methods for detecting carcinogenesis and mutagens with the Salmonella/mammalian microsome mutagenicity test. Mutation Res 31:347-364.

Anderson D, McGregor DB (1980): The effect of solvents upon the yield of revertants in the Salmonella/activation mutagenicity assay. Carcinogenesis 1:363-368.

Ashby J, Tennant RW (1988): Chemnical structure, Salmonella mutagenicity and extent of carcinogenicity as indicators of genotoxic carcinogenesis among 222 chemicals tested in rodents by the U.S. NCI/NTP. Mutat Res 204:17-115.

Awerbuch TE, Sinskey AJ (1980): Quantitative determination of half-lifetimes and mutagenic concentrations of chemical carcinogens using a diffusion bioassay. Mutation Res 74:125-143.

Balansky RM, Bryson L (1985): The stimulatory effect of potassium ions on MMU- and MNNG-induced mutagenesis in *Salmonella typhimurium* TA1535 and TA100. Neoplasma 32:313-321.

Barber ED, Donish WH, Mueller KR (1983): The relationship between growth and reversion in the Ames Salmonella plate incorporation assay. Mutat Res 113:89-101.

Bartsch H, Malaveille C, Camus AM, Master-Planche G, Brun G, Hautefeuille A, Sabadie N, Barbin A, Kuroki T, Drevon C, Piccoli C, Montesano R (1980): Validation and compositive studies on 180 chemicals on *S. typhimurium* strains and V79 Chinese hamster cells in the presence of various metabolizing systems. Mutation Res 76:1-50.

Belser WL, Shaffer SD, Bliss RD, Hynds PM, Yamamoto L, Pitts JN, Winer JA (1981): A standardized procedure for quantification of the Ames Salmonella/mammalian-microsome mutagenicity test. Environ Mut 3:123-139.

Brenner M, Ames BN (1971): The histidine operon and its regulation. In H.J. Vogel (ed.) "Metabolic pathways, Vol 5: Metabolic Regulation." New York, Academic Press, Inc., pp(349-387).

Burnett C, Fuchs C, Corbett J, Menkart J (1982): The effect of dimethylsulfoxide on the mutagenicity of the hair dye P-phenylenediamine. Mutation Res 103:1-4.

Castonguay A, Pepin B, Brien N (1991): Modulation of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone demethylation and denitrosation by rat liver microsome. Cancer Lett 59:67-74.

Cheli C, DeFrancesco D, Petrullo LA, McCoy EC, Rosenkranz HS (1980): The Salmonella mutagenicity assay: reproducibility. Mutation Res 74:145-150.

Claxton LD, Allen J, Auletta A, Mortelmans K, Nestmann E, Zeiger E (1987): Guide for the *Salmonella typhimurium*/mammalian microsome tests for bacterial mutagenicity. Mutation Res 189:83-91.

Claxton LD, Houk VS, Allison JC, Creason J (1991a): Evaluation the relationship of metabolic activation system concentrations and chemical dose concentration for the Salmonella spiral and plate assays. Mutation Res 253:127-136.

Claxton LD, Houk VS, Monteith LG, Myers LE, Hughes TJ (1991b): Assessing the use of known mutagens to calibrate the *Salmonella typhymurium* mutagenicity assay: I. Without exogenous activation. Mutation Res 253:137-147.

Claxton LD, Houk VS, Warner JR, Myers LE, Hughes TJ (1991c): Assessing the use of known mutagens to calibrate the *Salmonella typhimurium* mutagenicity assay: II. With exogenous activation. Mutation Res 253:149-159.

Claxton LD, Stead AG, Walsh D (1988): An analysis by chemical class of Salmonella mutagenicity tests as predictors of animal carcinogenicity. Mutation Res 205:197-225.

Commoner B, Vithayathil AJ, Dolara P, Nair S, Madyastha P, Cuca GG (1978): Formation of mutagens in beef and beef extract during cooking. Science 201:913-916.

Conney AH (1982): Induction of microsomal enzymes by foreign chemicals and carcinogens by polycyclic aromatic hydrocarbons: G.H.A. Clowes Memorial Lecture. Cancer Res 42:4875-4917.

Crespi CL, Penman BW, Gelboin HV, Gonzalez FJ (1991): A tobacco smoke-derived nitrosamine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone is activated by multiple human cytochrome P450's including the polymorphic human cytochrome P450 2D6. Carcinogenesis 12:1197-1201.

deFlora S, Zanacchi P, Camoirano A, Bennicelli C, Badolati GS (1984): Genotoxic activity and potency of 135 compounds in the Ames reversion test and in a bacterial DNA-repair test. Mutat Res 133:161-198.

deRaat WK, Willems MI, deMeijene FA (1984): Effects of amount and type of agar on the number of spontaneous revertants in the Ames test. Mutation Res 137:33-37.

deSerres FJ, Shelby MD (1979): Recommendations on data production and analysis using the Salmonella/microsome mutagenicity assay. Mutation Res 64:159-165.

Dolara P (1982): Beef extract mutagens, and not liver S9, increase mutation frequency in the Ames test. Mutation Res 104:345-346.

Emi Y, Chijiiwa C, Omura T (1990): A different cytochrome P450 form is induced in primary cultures of rat hepatocytes. Proc Natl Acad Sci 87:9746-9750.

Forster R, Green MHL, Priestley (1980a): Optimal levels of S9 fraction in the Ames fluctuation tests: apparent importance of diffusion of metabolites from top agar. Carcinogenesis 1: 337-346.

Forster R, Green MHL, Priestley (1980b): Optimum S9 concentrations in bacterial mutagenicity assays. Advances in Experimental Medicine and Biology 163(B):1263-1269.

Friederich U, Aeschbacher HU, Seiler JP, Würgler FE (1982): The Salmonella/microsome assay: some possible causes for interlaboratory variations. Mutation Res 103:133-140.

Gatehouse D (1987): Guidelines for testing environmental agents: Critical features of bacterial mutation assays. Mutagenesis 2(5):397-409.

Gatehouse D, Wedd DJ (1984): The differential mutagenicity of isoniazid in fluctuation assays and Salmonella plate tests. Carcinogenesis 5(3):391-397.

Georghiou PE, Blagden PA, Winsor L, Williams DT (1989): Spontaneous revertants in modified *S. typhimurium* mutagenicity tests employing elevated numbers of test strains. Mutation Res 225:33-39.

Glatt H, Martiné U, Baidossi W, Blum J (1991): Strong influence of the exposure medium on mutagenicity in the Ames test: 7-methylbenz[a]anthracene-5,6-oxide. Mutagenesis 6(6):537-540.

Göggelmann W (1980): Factors modulating mutagenicity in the Ames test. Mutation Res 74:208.

Grafe A, Mattern IE, Green M (1981): A European collaborative study of the Ames assay. I. Results and general interpretation. Mutation Res 85:391-410.

Guengerich FP (1991): Oxidation of toxic and carcinogenic chemicals by human P450 enzymes. Chem Res Toxicol 4:391-407.

Guo Z, Smith TJ, Thomas PE, Yang CS (1991): Metabolic activation of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone as measured by alkylation *in vitro* and its inhibition by isothiocyanates. Cancer Res 51:4798-4803.

Guttenplan JB (1980): Enhanced mutagenic activities of N-nitroso compounds in weakly acidic media. Carcinogenesis 1:439-444.

Hecht SS, Hoffmann (1988): Tobacco-specific nitrosamines, an important group of carcinogens in tobacco and tobacco smoke. Carcinogenesis (Lond) 9:875-884.

Josephy PD (1989): New developments in the Ames Assay: High-sensitivity detection of mutagenic arylamines. Bioessays 11(4):108-111.

Keir LE, Brusick DJ, Auletta AE, Vonltalle ES, Brown MM, Simmon VF, Dankel V, McCann J, Mortelmans K, Prival M, Rao TK, Rag V (1986): The *Salmonella typhimurium*/mammalian microsomal assay. A report of the U.S. Environmental Protection Agency Gene-Tox Program. Mutation Res 168:69-240.

Langer PJ, Shanabruch WG, Walker GC (1981): Functional organization of plasmid pKM101. J Bacteriol 145:1310-1316.

Lowry OH, Rosebraugh NJ, Far AL, Randall KJ (1951): Protein measurement with the folin phenol reagent. J Biol Chem 193:265-275.

Majeska JB, McGregor DB (1990): Effect of minimal plate preparation on the mutagenic activity of 4-[2(aryl)ethenyl]-2,6-dimethylphenols in the Ames assay. Environ Mol Mutagen 15:39.

Majeska JB, McGregor DB (1992): Effects of plate preparation on results in microbial mutation assays. Environ Mol Mutagen 19:244-252.

Maron D, Katzenellenbogen J, Ames BN (1981): Compatability of organic solvents with the Salmonella/microsome test. Mutation Res 88:343-350.

Maron DM, Ames BN (1983): Revised methods for the Salmonella mutagenicity test. Mutation Res 113:173-215.

McCann J, Springarn NE, Kobori J, Ames BN (1975): Detection of carcinogens as mutagens: bacterial tester strains with R factor plasmids. Proc Natl Acad Sci (USA) 72:979-983.

McCoy EC, Hyman J, Rosenkranz HS (1979): Conversion of environmental pollutants to mutagens by visible light. Biochem Biophys Res Commun 89:729-734.

Myers LE, Adams NH, Hughes TJ, Williams LR, Claxton LD (1987): An interlaboratory study of an EPA/Ames/Salmonella test protocol. Mutation Res 182:121-133.

Nebert DW, Gonzalez FJ (1987): P450 genes: Structure, evolution and regulation. Ann Rev Biochem 56:945-993.

Neudecker T, Eder E, Deininger C, Henschler D (1989a): Crotonaldehyde is mutagenic in *Salmonella typhimurium* TA100. Environ Mol Mutagenesis 14:146-148.

Neudecker T, Eder E, Deininger C, Hoffman C, Henschler D (1989b): Mutagenicity of methylvinyl ketone in *S. typhimurium* TA100 - an indication for epoxidation as an activation mechanism. Mutation Res 227:131-134.

O'Brien K, Moss E, Judah D, Neal G (1983): Metabolic basis of the species differences to aflatoxin B_1 induced hepatotoxicity. Biochem Biophys Res Commun 114:813-821.

Okey AB (1990): Enzyme induction in the cytochrome P-450 system. Pharmac Ther 45:241-298.

Paolini M, Sapigni E, Hrelia P, Scotti M, Morotti M, Cantelli-Forti G (1991): Wide spectrum detection of precarcinogens in short-term bioassays by simultaneous superinduction of multiple forms of cytochrome P450 isoenzymes. Carcinogenesis 12:759-766.

Peak MJ, Dornfeld SS, Venters D (1982): Liver-microsome S9 enzyme increases spontaneous background mutation frequency in the Ames Salmonella test system in the absence of any added mutagen. Mutation Res 103:263-265.

Peak MJ, Hass B, Dornfeld SS (1980): The Salmonella mutagenicity assay: reproducibility. Mutation Res 74:399-400.

Rossman TG, Molina M (1983): Rat liver S9 preparations contain comutagenic activity. Mutation Res 122:103-109.

Safe S, Bandiera S, Sawyer T, Robertson L, Safe L, Parkinston A, Thomas PE, Ryon DE, Reik LM, Levin W, Denomme MA, Fujita T (1985): PCB's: structure-function relationships and mechanism of action. Environ Health Perspect 60:47-56.

Salmeen I, Durisin AM (1981): Some effects of bacteria population on quantitation of Ames Salmonella-histidine reversion mutagenesis assays. Mutation Res 85:109-118.

Seiler JP, Mattern IE, Green MHL, Anderson D (1980): Second European workshop on bacterial in vitro mutagenicity test systems (Ames test meeting 1979). Mutation Res 74:71-75.

Shanabruch WG, Walker GC (1980): Localization of the plasmid (pKM101) gene(s) involved in *recA*$^+$ *lexA*$^-$ - dependent mutagenesis. Mol Gen Genet 179:289-297.

Shirai A, Umezawa K, Matsushima T, Sugimura T (1980): Enhancement of the mutagenicities of N-methyl-N-nitro-N-nitrosoguanidine and N-methyl-N-nitrosourea by glucose. Mutat Res 72:73-77.

Speck WT, Rosenkranz HS (1975): Base substitution mutations induced in Salmonella strains by visible light. Photochem Photobiol 21:369-371.

Stead AG, Hasselbland V, Creason JP, Claxton L (1981): Modeling the Ames test. Mutation Res 85:13-27.

Szybalski W (1958): Special microbiological systems. II. Observations on chemical mutagenesis in microorganisms. Ann NY Acad Sci 76:475-489.

Teel RW (1992): Effects of different inducers of cytochrome P450 on the mutagenesis of the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in *Salmonella typhimurium* TA1535. Anticancer Res 12:1287-1290.

Vithayathil AJ, Commoner B, Nair S, Madyastha P (1978): Isolation of mutagens from bacterial nutrients containing beef extract. J Tox Environ Health 4:189-202.

Vogel HJ, Bonner DM (1956): Acetylornithinase of *Escherichia coli*: partial purification and some properties. J Biol Chem 218:97-106.

Walker GC, Dobson PP (1979): Mutagenesis and repair deficiencies of *Escherichia coli umuC* mutants are suppressed by the plasmid pkm101. Mol Gen Genet 172:17-24.

Wheeler L, Halala M, Demeo M (1981): A comparison of aflatoxin B_1-induced cytotoxicity, mutagenicity and prophage induction in *Salmonella typhimurium* mutagen tester strains TA1535, TA1538, TA98 and TA100. Mutat Res 83:39-48.

Witmer C, Cooper K, Kelly J (1982): Effects of plating efficiency and lowered concentration of salts on mutagenicity assays with Ames' Salmonella strains. Advances in Experimental Medicine and Biology 136B:1271-1284.

Yahagi TM, Degawa M, Seino Y, Matsushima T, Nagao M, Sugimura T, Hashimoto Y (1975): Mutagenicity of carcinogenic azo dyes and their derivatives. Cancer Lett 1:91-96.

Yahagi TM, Nagao M, Seino Y, Matsashima T, Sagimara T, Okada M (1977): Mutagenicities of N-nitrosamines on Salmonella. Mutation Res 48:121-130.

Yoo JSH, Ishizaki H, Yang C (1990): Roles of cytochrome P50 IIE1 in the dealkylation and denitrosation of N-nitrosodimethylamine and N-nitrodiethylamine in rat liver microsomes. Carcinogenesis 11:2239-2243.

Zang Z, Hamilton SM, Stewart C, Strother A, Teel RW (1993): Inhibition of liver microsomal cytochrome P450 activity and metabolism of the tobacco-specific nitrosamine NNK by capsaicin and ellagic acid. Anticancer Res 13:2341-2346.

CHAPTER III

MODULATION OF THE MUTAGENICITY AND METABOLISM OF THE TOBACCO-SPECIFIC NITROSAMINE 4-(METHYLNITROSAMINO)-1-(3-PYRIDYL)-1-BUTANONE (NNK) BY PHENOLIC COMPOUNDS

Cecil H. Miller[1], Andre Castonguay[2], and Robert W.Teel[3]

[1]Graduate student that conducted the experiments, collected the data and helped prepare the manuscript

[2]Professor that we collaborated with who did the Western blot analysis and helped prepare the manuscript

[3]Student's research committee chairmen who supervised the research and helped prepare the manuscript

Environmental and Molecular Mutagenesis (submitted)

MODULATION OF THE MUTAGENICITY AND METABOLISM OF THE TOBACCO-SPECIFIC NITROSAMINE 4-(METHYLNITROSAMINO)-1-(3-PYRIDYL)-1-BUTANONE (NNK) BY PHENOLIC COMPOUNDS

Cecil H. Miller[1], Andre Castonguay[2], and Robert W.Teel[1]

[1]Department of Physiology and Pharmacology,
School of Medicine
Loma Linda University
Loma Linda, CA 92340 (USA)

and

[2]Laboratory of Cancer Etiology and Chemoprevention,
School of Pharmacy
Laval University
Quebec City G1Y 7P4 (Canada)

Key words: Phenols; mutagenicity; metabolism; tobacco nitrosamines; P-450 inducers

Correspondence: Dr Robert W. Teel
Department of Physiology and Pharmacology
School of Medicine
Loma Linda University
Loma Linda, CA 92350

SUMMARY

NNK is an important environmental carcinogen generated during tobacco smoking. The carcinogenic response to tobacco smoking is modulated by nutritional factors. In this study, we compared the effects of phenobarbital and ß-naphthoflavone, two P-450 inducers, on the mutagenicity (*Salmonella typhimurium* TA1535) and the metabolism of NNK by hamster liver microsomes. Western analysis of microsomal proteins revealed an increased expression of protein recognized by polyclonal antibodies specific for P-450 1A2 and P-450 2B1/2B2. Presence of these proteins significantly increased the mutagenicity of NNK. Methyl hydroxylation of NNK was approximately 40- fold greater than α-methylene hydroxylation. The inhibition of mutagenicity and metabolism by polyphenolic compounds were correlated to some extent. Inhibition of mutagenesis by (+)-catechin, ellagic acid, esculetin, (-) esculin and propyl gallate was dependant on the enzyme induction treatment. While ellagic acid was the most efficient inhibitor with control microsomes, propyl gallate was the most efficient with phenobarbital and ß-naphthoflavone induced microsomes suggesting that phenolic

compounds have selective affinities for specific P-450 isozymes activating NNK.

INTRODUCTION

The nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) is formed by nitrosation of nicotine during tobacco processing [Burton et al., 1989] and is transferred to the smoke during burning of cigarettes [Fisher et al., 1990a]. It is estimated that the American smoker is exposed to an average of 2 μg of NNK daily [Fischer et al., 1990b]. NNK is also present in sidestream smoke, an important part of environmental tobacco smoke to which non-smokers are exposed [Brunnemann et al., 1992]. The high carcinogenic potency of NNK in three species of laboratory animals and its remarkable specificity for lung tissues [Hecht and Hoffmann, 1988] have lead to the conclusion that NNK is an important etiological factor in lung cancer in smokers [Hecht and Hoffmann, 1989].

NNK requires activation by cytochrome P-450 to exhibit its mutagenic and carcinogenic properties [Crespi et al., 1991; Hecht and Hoffmann, 1988]. The initial and crucial step of this activation is an α-hydroxylation of the two carbons bound to the N-nitroso group. This forms reactive electrophilic species that methylate and pyridyloxobutylate purine bases of DNA [Murphy et al., 1990].

Several cytochrome P-450 isozymes have been associated with the metabolic activation of NNK including P-450 2B1, 1A1, 1A2, P-450 NMa, P-450 NMb [Devereux et al., 1988; Smith et al., 1992; Guo et al., 1991; Hong et al., 1992] and human P-450 2D6 and 2E1 [Crespi et al., 1991; Yamazaki et al., 1992]. The distribution of these isozymes is species and tissue specific [Adesnik and Atchinson, 1985].

Nutrition is known to affect the tumorigenic response to tobacco smoking [Hirayama, 1984]. This reflects the close relationship of diet and xenobiotic metabolism [Yang et al., 1992]. Accordingly, it is important to identify chemical compounds of natural or synthetic origin that can inhibit NNK mutagenicity and carcinogenicity by modulating its metabolism. Phenolic compounds are widely distributed in various foods present in the normal human diet [Haslam, 1981]. Previous studies from our laboratories have shown that phenolic compounds inhibit NNK carcinogenesis [Pepin et al., 1990]. Other investigators have described the antimutagenic action of (+)-catechin, ellagic acid and propyl gallate [Mandal et al., 1987; Nagabhushan and Bhide, 1988; Newmark, 1984; Wood et al., 1982].

Considering the strong causal link between smoking or chewing tobacco and cancer and the presence of NNK in tobacco and tobacco smoke, it is important to define the relative importance of each P-450 isoform in the activation of NNK. The aim of this study was to compare the efficacies of phenolic compounds to inhibit the activation of NNK to reactive mutagenic intermediates. We selected five phenolic compounds, (+)-catechin, ellagic acid, esculetin, (-) esculin and propyl gallate which are present in the human diet or medicinal plants. P-450 isoforms were induced selectively with phenobarbital or ß-naphthoflavone. The information obtained is valuable in predicting the efficacies of phenols and organs to be targeted by these compounds in laboratory chemoprevention studies.

MATERIALS AND METHODS

Chemicals

$[5\text{-}^3H]$ NNK (2.51 Ci/mmole) and unlabeled NNK (purity >97%) were purchased from Chemsyn Science Laboratory (Lenexa, KS). The synthesis of NNK metabolites used as reference standards for HPLC analysis has been reported [Hecht et al., 1980; Hecht et al., 1981]. Ellagic acid, NADP+, glucose-6-phosphate, glucose-6-phosphate dehydrogenase and dimethylsulfoxide (DMSO) were obtained from Sigma Chemical Co. (St. Louis, MO). (-)Esculin, esculetin, propyl gallate and (+)-catechin (98% purity) were obtained from Aldrich Chemical Co. (Milwaukee, WI). Ellagic acid was recrystallized in pyridine [Pepin et al., 1990]. Bactoagar was obtained from VWR Scientific (Cerritos, CA) and nutrient broth No. 2 was purchased from Oxoid Co. (Basingstoke, Hants, UK).

Animals

Male Syrian golden hamsters, 150-160 g (Charles River Laboratory, Wilmington, ME) were given tap water and Purina rodent chow *ad libitum*. Two groups of four hamsters were injected

i.p. with phenobarbital in saline (60 mg/kg) or ß-naphthoflavone in olive oil (80 mg/kg) daily for five days [Paolini et al., 1991]. Control animals were given daily injections of saline. All hamsters were sacrificed by cervical dislocation, 24 hrs after the last injection.

Preparation of microsomes

The livers were removed aseptically and homogenized in two volumes of 0.25 M Tris-HCl (pH 7.4) containing 0.15 M sucrose and 1 mM EDTA. Microsomal fractions were obtained by differential centrifugation of the homogenate [Maron and Ames, 1983] and resuspended in Tris-HCl:sucrose:EDTA buffer. Microsome fractions from each group of four hamsters were pooled, aliquoted and stored at -80°C. Protein content was determined according to Lowry et al [1951].

Electrophoresis, immunoblotting and immunostaining

Electrophoresis of microsomal proteins was carried out on 7.5% gels in the presence of sodium dodecyl sulfate. Samples were mixed in Laemmli buffer containing ß-mercaptoethanol. Transfer to

nitrocellulose and immunostaining using goat anti-rabbit (dilution 1/100), rabbit anti-goat IgG (dilution 1/100) and goat peroxidase-immunoperoxidase (dilution 1/3000, Organon Teknika Corp., West Chester, PA) has been described [Domin et al., 1984; Tynes & Philpot, 1987]. The specificities of the goat anti-rabbit P-450 1A and 2B antibodies have been documented [Domin and Philpot, 1986; Serbjit-Singh et al., 1979].

Assay of NNK mutagenesis

Nontoxic concentrations of the five phenolic compounds had been determined previously [Teel and Castonguay, 1992]. The inhibition of NNK mutagenicity by these compounds was determined as follows. Sterile tubes containing 1 mg microsomal protein from saline-, phenobarbital- or ß-naphthoflavone-treated hamsters, 100 µl of 80 mM solution of unlabeled NNK in saline, cofactor buffer (pH 6.8) [Guttenplan, 1980], 0.15 ml of exponentially growing (5 hr culture) *Salmonella typhimurium* strain TA1535 and 0, 0.1, 0.2 or 0.4 µmoles (-) esculin, esculetin, propyl gallate, (+)-catechin or ellagic acid in a final volume of one ml were incubated in triplicate in a shaking

water bath at 37°C for 30 min. The final volume of DMSO (50 µl) was uniform in all incubations to correct for its reported inhibitory effect [Teel, 1992; Lord et al., 1989]. Two ml of top agar was added to each tube and the resulting mixture was poured onto minimal essential agar plates [Maron and Ames, 1983]. The plates were incubated at 37°C for 48 hr before counting the histidine-independent (His^+) colonies. Results were expressed as the mean number of NNK-induced His^+ revertant colonies/plate. Spontaneous revertant colonies (9-12/plate) were subtracted.

Assay of NNK metabolism

The effect of phenols on the microsome mediated metabolism of NNK was performed as a modification of a described procedure [Smith et al., 1990]. The incubation mixture contained 5 mM glucose-6-phosphate, 1.5 units glucose-6-phosphate dehydrogenase, 1 mM NADP+, 1 mM EDTA, 3 mM $MgCl_2$, 12.8 µCi [5-^{3}H]NNK, 0.4 µmole phenolic compound ((+)-catechin, ellagic acid, esculetin, (-) esculin, propyl gallate) in 50 µl DMSO and 5 mM sodium bisulfite in a final volume of 1 ml. Following incubation at 37°C for 10 min, the reaction

was started by addition of 1 mg microsomal protein. Blanks were incubated with boiled microsomes. The mixture was incubated for 30 min in a shaking water bath at 37°C and stopped by the addition of 200 μl each of 25% zinc sulfate and saturated barium hydroxide. The precipitated samples were centrifuged at 14,000 xg for 20 min and filtered through 0.45 μm filters (MSI, Westboro, MA). One hundred μl of the filtrate and 7 μl of NNK metabolite standards were coinjected onto a reversed-phase HPLC system using a μBondapak C18 column (3.9 mm x 300 mm; Waters, Milford, MA). NNK and its metabolites were eluted with sodium acetate buffer (pH 6) and methanol [Peterson et al., 1991a]. Keto aldehyde-bisulfite adducts were stabilized by the addition of 1 mM sodium bisulfite to the elution buffer. The eluate was monitored at 254 nm and 1 ml fractions were combined to five ml of scintillation cocktail. (Scintiverse LC, Fisher Scientific, Tustin, CA). Radioactivity was determined by liquid scintillation spectroscopy (Beckman LS5801 counter, Beckman Instrument Division, Berkeley, CA) and was expressed as pmoles/mg microsome protein/min. Recovery of total radioactivity during HPLC analysis was >70%.

Statistical Analysis

Data were analyzed by the student's unpaired t-test using the MacIntosh Stat-View software program. Statistical significance was defined as $p < 0.05$ compared with control values.

RESULTS

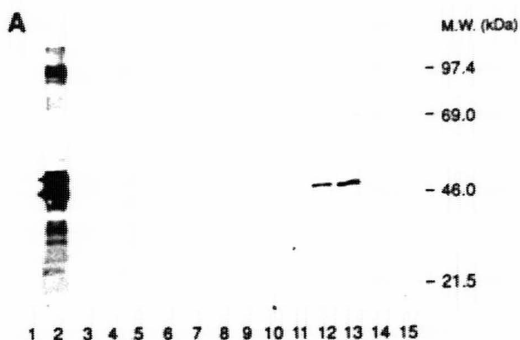
Western analysis of P-450

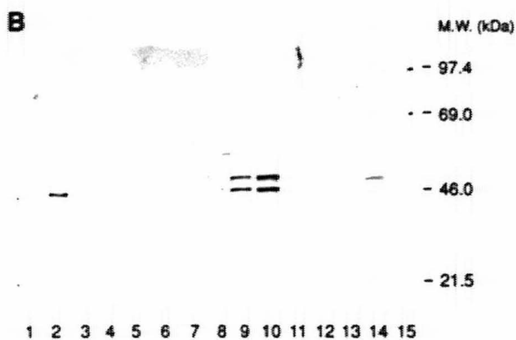
Orthologues of cytochrome P-450 were detected on immunoblots of liver microsomes using goat anti-rabbit P-450 1A2 and 2B (Figure 1). These three enzymes were present in liver microsomes from TCDD-treated rabbit. The orthologue 2B1 was also detected in lung microsomes from rat (Figure 1, Panel B). In liver microsomes, the orthologue 1A2 which was detected in saline-treated (control) hamsters was increased significantly by ß-naphthoflavone (Figure 1, Panel A). The orthologues 2B1 and 2B2 were not detected in liver of saline-treated (control) hamsters. Two bands, corresponding most likely to 2B1 and 2B2, were present in phenobarbital-treated hamsters.

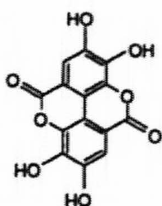
Mutagenesis of NNK

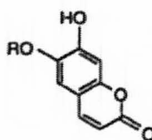
The structures of the five phenolic compounds selected for this study are shown in Figure 2. All five compounds have a 1,2-dihydrobenzene (catechol) structure element. Ellagic acid, esculetin and (-)esculin have a lactone structure element. Ellagic acid

Figure 1: Immunoblots of P-450 monooxygenases with goat anti-rabbit 1A1 (Panel A) or 2B1/2B2 (Panel B). Lanes 1 and 15: molecular weight markers; Lane 2: 10 µg of liver microsomes from TCDD-treated rabbit,; Lanes 3 and 7: blanks; Lanes 4, 5 and 6: 2, 5 and 10 µg respectively of liver microsomes from saline-treated hamsters; Lanes 8, 9 and 10: 2, 5 and 10 µg respectively of liver microsomes from phenobarbital-treated hamsters; Lanes 11, 12 and 13: 2, 5 and 10 µg respectively of liver microsomes from ß-naphthoflavone-treated hamsters; Lane 14: 50 µg of rat lung microsomes.


A
M.W. (kDa)
- 97.4
- 69.0
- 46.0
- 21.5
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15


B
M.W. (kDa)
- 97.4
- 69.0
- 46.0
- 21.5
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

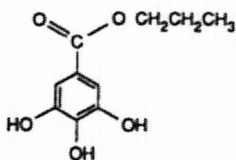


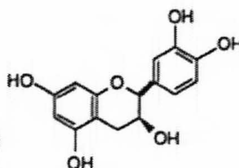


ELLAGIC ACID

R = H **ESCULETIN**

R = β - D - Glu **ESCULIN**





PROPYL GALLATE

(+)-CATECHIN

Figure 2: Molecular structures of the phenolic compounds ellagic acid, esculetin, (-)esculin, propyl gallate, and (+)-catechin.

and propyl gallate are both derived from gallic acid. Mutagenicity of NNK was tested in *Salmonella typhimurium* TA1535. The effects of (+)-catechin, ellagic acid, esculetin, (-)esculin and propyl gallate at concentrations of 0, 0.1, 0.2 and 0.4 μmoles/plate on the mutagenicity of NNK activated by liver microsomes of saline-treated hamster are shown in Figures 3A-E. All the phenols except (+)-catechin, significantly inhibited the mutagenicity of NNK. The most effective inhibitor with this activating system was ellagic acid.

Figures 4A-E shows the effects of the five phenols on the mutagenicity of NNK activated by microsomes from phenobarbital-treated hamsters. (-)Esculin was the only phenol lacking significant inhibitory activity. (+)-Catechin and esculetin were weak inhibitors. The inhibitory effects of ellagic acid and propyl gallate on the mutagenicity of NNK were comparable.

As shown in Figures 5A-E, (+)-catechin and (-)esculin did not inhibit the mutagenicity of NNK activated by microsomes from ß-naphthoflavone-treated hamsters. Propyl gallate was the strongest inhibitor. Esculetin and ellagic acid showed a similar pattern of

Figure 3: Effect of different concentrations of five phenolic compounds on the mutagenesis of NNK in *Salmonella typhimurium* TA1535 mediated by noninduced hamster liver microsomes. Procedural details for performing the experiments are given in Material and Methods. Asterisks indicate statistically significant difference from control (0 μmole/plate), $p < 0.05$.

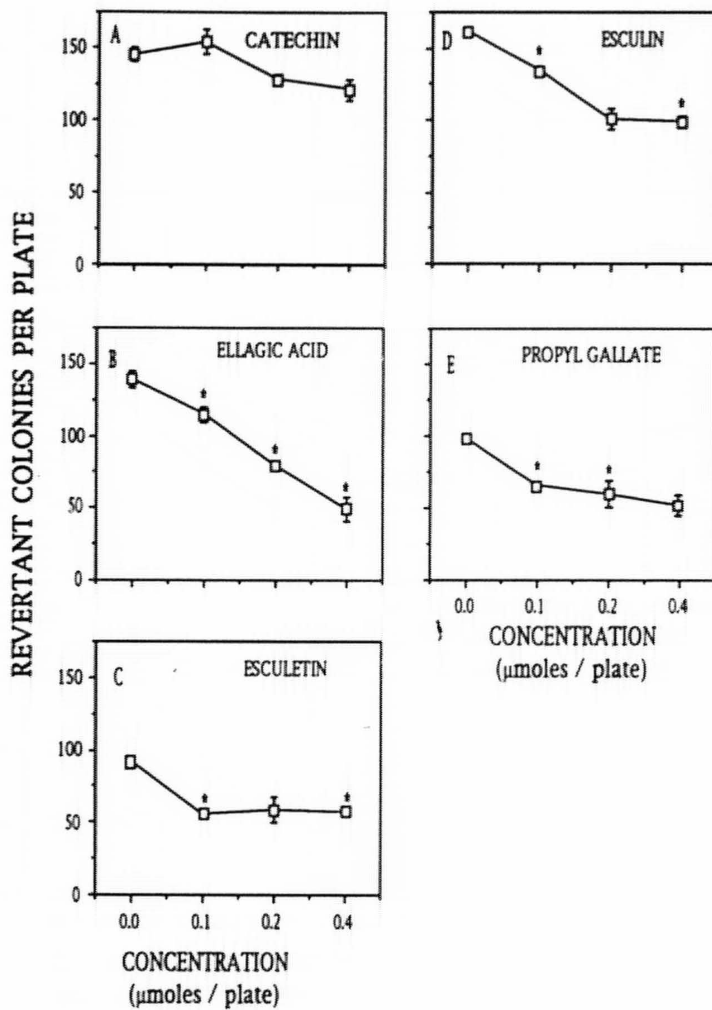
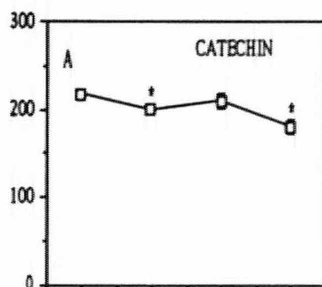
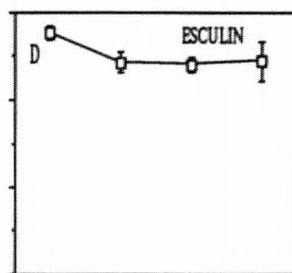
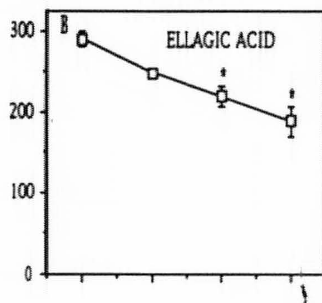

REVERTANT COLONIES PER PLATE
A
CATECHIN
B
ELLAGIC ACID
C
ESCULETIN
D
ESCULIN
E
PROPYL GALLATE
150
100
50
0
0.0
0.1
0.2
0.4
CONCENTRATION
(µmoles / plate)

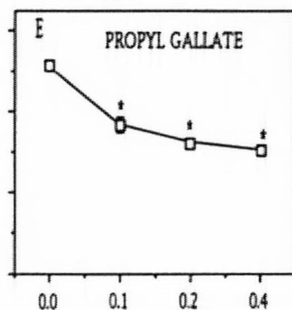
Figure 4: Effect of different concentrations of five phenolic compounds on the mutagenesis of NNK in *Salmonella typhimurium* TA1535 mediated by phenobarbital-induced hamster liver microsomes. Procedural details for performing the experiments are given in Materials and Methods. Asterisks indicate statistically significant difference from control (0 μmole/plates), $p < 0.05$.

REVERTANT COLONIES PER PLATE









CONCENTRATION
(μmoles / plate)

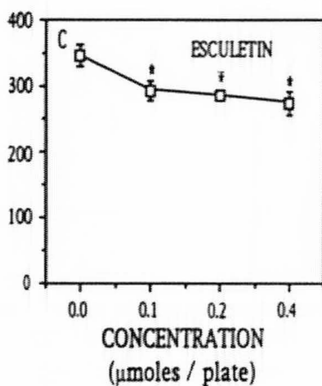
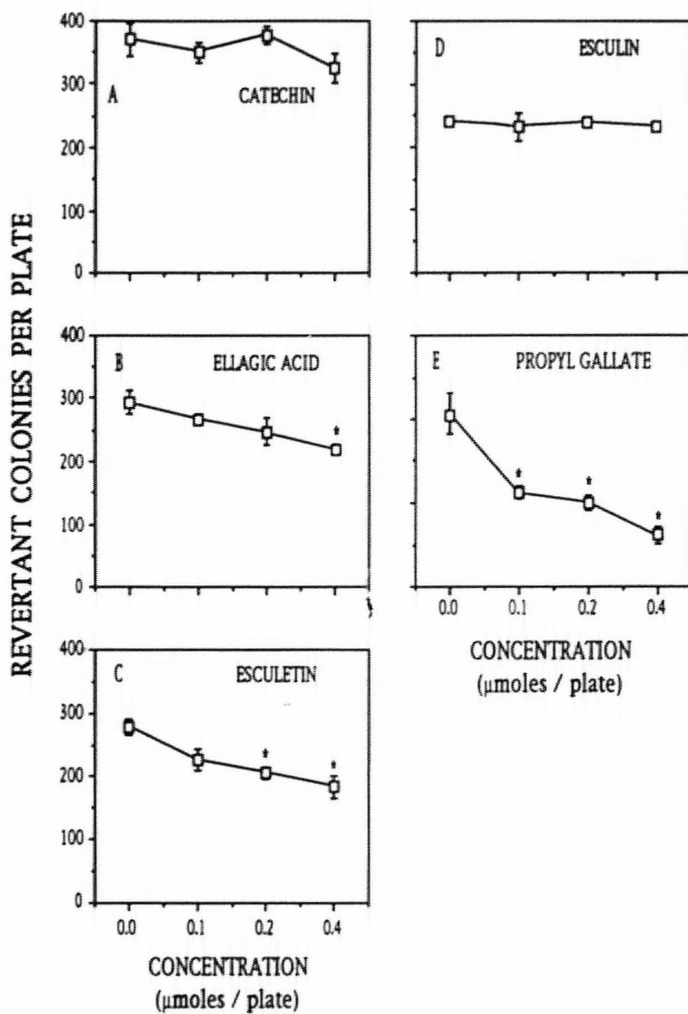


Figure 5: Effect of different concentrations of five phenolic compounds on the mutagenesis of NNK in *Salmonella typhimurium* TA1535 mediated by ß-naphthoflavone-induced hamster liver microsomes. Procedural details for performing the experiments are given in Materials and Methods. Asterisks indicate statistically significant difference from control (0 μmole/plate), $p < 0.05$.


REVERTANT COLONIES PER PLATE
A
CATECHIN
D
ESCULIN
B
ELLAGIC ACID
E
PROPYL GALLATE
C
ESCULETIN
400
300
200
100
0
0.0
0.1
0.2
0.4
CONCENTRATION
(μmoles / plate)

inhibition although inhibition was significant only at the higher concentrations tested.

Figure 6 shows the effects of treatment with phenobarbital and ß-naphthoflavone on the mutagenesis of NNK by hamster microsomes. Both phenobarbital and ß-naphthoflavone treatment produced a significant increase in the mutagenesis of NNK compared to the saline-injected animals.

Metabolism of NNK

The pathways in the metabolism of NNK are shown in Figure 7. Carbonyl reduction of NNK yielded the N-nitroso alcohol NNAL (pathway a). N-oxidation of the pyridine ring of NNK and NNAL yields NNK-N-oxide and NNAL-N-oxide respectively (pathway b). While methyl hydroxylation produces keto alcohol, the keto aldehyde originates from α-methylene hydroxylation (pathway c). The addition of 1 mM sodium bisulfite in the incubation mixture and HPLC eluant forms and stabilizes the keto aldehyde as a keto aldehyde-bisulfite adduct [Peterson et al., 1991a]. The subsequent

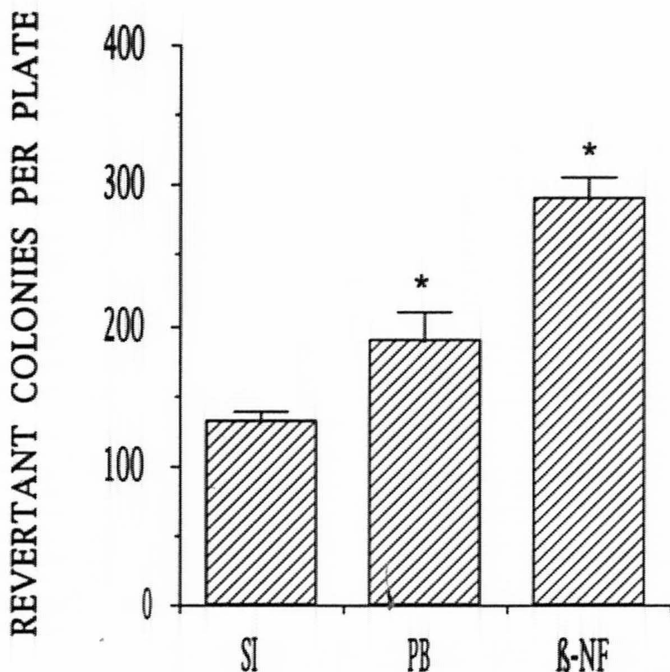


Figure 6: Effect of phenobarbital (PB) and ß-naphthoflavone (ß-NF) treatment on the mutagenicity of NNK in *Salmonella typhimurium* TA1535 mediated by hamster liver microsomes. Procedural details for treatment and performing mutagenesis assays are described in Materials and Methods. Asterisks indicate statistically significant difference frome saline-injected (SI) control, $p < 0.05$.

Figure 7: Metabolic pathways of NNK: carbonal reduction (pathway a), pyridine N-oxidation (pathway b), and α-carbon hydroxylation (pathway c).
Metabolites: NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol;
NNK-N-oxide, 4-(methylnitrosamino)-1-(3-pyridyl-N-oxide)-1-butanone;
NNAL-N-oxide, 4-(methylnitrosamino)-1-(3-pyridyl-N-oxide)-1-butanol;
keto alchol, 4-oxo-4-(3-pyridyl)-1-butanol;
hydroxy acid, 4-hydroxy-4-(3-pyridyl) butyric acid;
keto aldehyde, 4-oxo-4-(3-pyridyl) butanal;
keto acid, 4-oxo-4-(3-pyridyl) butyric acid;
diol, 4-hydroxy-1-(3-pyridyl)-1-butanol.
The structures in brackets are hypothetical intermediates.
(Adapted from Jorquera et al., 1992; Hecht et al., 1980).

NNK-N-Oxide

NNK

NNAL

NNAL-N-Oxide

Keto aldehyde

Keto alcohol

Keto acid

Hydroxy acid

Diol

oxidation/reduction of keto alcohol and keto aldehyde yield the metabolites keto acid, hydroxy acid and diol.

The levels of metabolites formed by liver microsomes from saline-treated, phenobarbital-treated and ß-naphthoflavone-treated hamsters are shown in Table 1. Keto alcohol and NNAL were the two major metabolites formed by microsomes from saline-treated hamsters. Carbonyl reduction (pathway a, Figure 7) accounted for 44% of the total metabolism of NNK by these microsomes. Methyl and α-methylene hydroxylation (pathway c, Figure 7) accounted for 40%. N-oxidation of the pyridine ring (pathway b, Figure 7) represents only 15% of total NNK metabolism. This compares with 40%, 59% and 12% by phenobarbital-induced microsomes and 21%, 48% and 31% by ß-napthoflavone-induced microsomes.

Table 2 shows the effect of the five phenols on NNK metabolism by microsomes from saline-treated hamsters. We observed that (+)-catechin significantly increased α-carbon hydroxylation (metabolites keto acid and keto aldehyde) and slightly decreased carbonyl reduction (metabolite NNAL). Ellagic acid and esculetin inhibited α-carbon hydroxylation (with the exception of the effect of

Table 1. Effects of saline, phenobarbitol and ß-naphthoflavone treatment on the metabolism of NNK by hamster liver microsomes.

METABOLITE[a]	TREATMENT[b]		
ρmol/mg/min[c]	Saline	Phenobarbitol	ß-naphthoflavone
Hydroxy acid (c)	0.8 ± 0.1	N.D.	1.0 ± 0.4
Keto acid (c)	3.1 ± 0.5	4.2 ± 0.9	12.9 ± 3.3
Diol (c)	9.2 ± 1.2	11.9 ± 0.2	4.8 ± 1.2*
Keto alcohol (c)	38.1 ± 4.9	44.5 ± 0.8	38.2 ± 1.8
Keto aldehyde[d] (c)	0.8 ± 0.1	0.6 ± 0.1	1.4 ± 0*
NNAL-N-oxide (b)	4.7 ± 3.3	3.9 ± 0.2	24.2 ± 1.1*
NNK-N-oxide (b)	15.0 ± 1.8	3.3 ± 0.1*	13.2 ± 0.2
NNAL (a)	56.6 ± 0.2	44.9 ± 0.8*	25.6 ± 0.3*
Total (b)	19.6 ± 5.0	7.1 ± 0	37.4 ± 1.4
Total (c)	51.7 ± 6.7	61.4 ± 0.2	58.2 ± 3.1
% unmetabolized NNK + NNAL	45.5 ± 0.5	33.0 ± 0.8*	26.1 ± 0.5*

[a]Refer to Figure 7 for names of metabolites and metabolic pathways (given in parentheses).

[b]In vitro incubations were carried out as described in Materials and Methods.

[c]Values are the mean ± S.E. from two determinations. N.D. = not detected.

[d]Keto aldehyde was detected as the keto aldehyde-bisulfite adduct.

*Significantly different ($p \leq 0.05$) from control value.

Table 2. Effects of phenolic compounds (0.4 μmole) on the metabolism of NNK by liver microsomes from saline-treated hamsters.

METABOLITE[a]	TREATMENT[b]					
pmol/mg/min[c]	Control	(+)-Catechin	Ellagic Acid	Esculetin	(-)Esculin	Propyl gallate
Hydroxy acid (c)	0.8 ± 0.1	N.D.*	N.D.*	N.D.*	0.1 ± 0.1*	0.1 ± 0.005*
Keto acid (c)	3.1 ± 0.5	16.8 ± 1.8*	1.7 ± 0.03	18.0 ± 4.3	19.0 ± 0.6*	25.1 ± 0.9*
Diol (c)	9.2 ± 1.2	8.0 ± 0.7	6.7 ± 0.1	1.9 ± 0.9*	7.0 ± 3.4	4.2 ± 0.3
Keto alcohol (c)	38.1 ± 4.9	25.7 ± 4.3	30.3 ± 2.0	18.1 ± 2.4*	22.4 ± 0.2	34.8 ± 3.8
Keto aldehyde[d] (c)	0.8 ± 0.1	1.2 ± 0.1*	N.D.*	N.D.*	0.5 ± 0.1*	0.3 ± 0.3
NNAL-N-oxide (b)	4.7 ± 3.3	18.5 ± 0.8	0.5 ± 0	5.9 ± 0.7	10.9 ± 1.2	8.6 ± 0.1
NNK-N-oxide (b)	15.0 ± 1.8	8.0 ± 0.2	N.D.*	2.7 ± 1.0	6.3 ± 2.2	4.1 ± 0.3*
NNAL (a)	56.6 ± 0.2	47.4 ± 0.7*	84.3 ± 0.3*	86.6 ± 0.2*	63.4 ± 0.7*	39.7 ± 0.6*
Total (b)	19.6 ± 5.0	26.5 ± 1.0	0.5 ± 0*	8.7 ± 1.6	17.1 ± 3.3	12.6 ± 0.4
Total (c)	51.7 ± 6.7	51.7 ± 1.95	38.8 ± 2.0	38.0 ± 1.0	49.0 ± 3.7	64.5 ± 5.4
% unmetabolized NNK + NNAL	45.5 ± 0.5	31.2 ± 0.5*	69.2 ± 0.4*	64.7 ± 0.4*	43.6 ± 1.0	34.6 ± 0.3*

[a]Refer to Figure 7 for names of metabolites and metabolic pathways (given in parentheses).

[b]In vitro incubations were carried out as described in Materials and Methods.

[c]Values are the mean ± S.E. from two determinations. N.D. = not detected.

[d]Keto aldehyde was detected as the keto aldehyde-bisulfite adduct.

*Significantly different ($p \leq 0.05$) from control value.

esculetin on keto acid), inhibited N-oxidation and increased carbonyl reduction (NNAL). (-)Esculin had an inhibitory effect on the formation of hydroxy acid and keto aldehyde but enhanced the amount of keto acid produced. (-)Esculin also caused a small increase in the amount of NNAL produced. Propyl gallate decreased the production of hydroxy acid, NNK-N-oxide and NNAL but increased the amount of keto acid formed by noninduced (saline-injected) hamster liver microsomes.

The effect of the five phenols on NNK metabolism by microsomes from phenobarbital-treated hamsters are shown in Table 3. (+)-Catechin enhanced both α-carbon hydroxylation and N-oxidation but inhibited NNAL formation (carbonyl reduction). Both ellagic acid and esculetin inhibited the amounts of diol and keto alcohol formed. Esculetin inhibited the formation of NNAL-N-oxide but increased NNK-N-oxide and NNAL production. The only significant effect of (-)esculin was on the increased production of NNK-N-oxide. Propyl gallate increased the formation of keto alcohol but inhibited the formation of NNK-N-oxide and NNAL.

Table 3. Effects of phenolic compounds (0.4 µmole) on the metabolism of NNK by phenobarbitol-induced hamster liver microsomes.

METABOLITE[a] pmol/mg/min[c]	TREATMENT[b]					
	Control	(+)-Catechin	Ellagic Acid	Esculetin	(-)Esculin	Propyl gallate
Hydroxy acid (c)	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Keto acid (c)	4.2 ± 0.9	7.9 ± 1.1*	0.9 ± 0.7	2.3 ± 0.2	4.2 ± 0.2	5.2 ± 0.8
Diol (c)	11.9 ± 0.2	18.1 ± 0.4*	2.2 ± 0.5*	8.0 ± 0.4*	10.9 ± 0.9	13.1 ± 2.6
Keto alcohol (c)	44.5 ± 0.8	44.6 ± 3.0	17.1 ± 4.0*	33.6 ± 0.9*	51.9 ± 4.3	73.2 ± 0.7*
Keto aldehyde[d] (c)	0.6 ± 0.1	1.0 ± 0.03*	0.1 ± 0.1	0.4 ± 0	0.7 ± 0.1	0.6 ± 0.6
NNAL-N-oxide (b)	3.9 ± 0.2	5.9 ± 0.4*	1.5 ± 0.4	2.4 ± 0*	4.1 ± 0.1	5.7 ± 3.9
NNK-N-oxide (b)	3.3 ± 0.1	7.6 ± 0*	6.2 ± 2.5	7.1 ± 0.1*	11.6 ± 0.1*	N.D.*
NNAL (a)	44.9 ± 0.8	28.8 ± 0.2*	45.4 ± 8.9	69.3 ± 0.3*	41.2 ± 0.05	24.8 ± 0.4*
Total (b)	7.1 ± 0.2	13.5 ± 0.4*	7.8 ± 2.9	9.4 ± 0.1*	15.8 ± 0.1*	7.8 ± 1.7
Total (c)	61.4 ± 0.2	71.5 ± 4.4	20.4 ± 4.2*	44.3 ± 0.3*	67.8 ± 5.4	92.0 ± 1.8*
% unmetabolized NNK + NNAL	33.0 ± 0.8	19.5 ± 0.1*	76.5 ± 1.8*	54.2 ± 0.4*	31.6 ± 0.6	20.7 ± 0.5*

[a]Refer to Figure 7 for names of metabolites and metabolic pathways (given in parentheses).

[b]In vitro incubations were carried out as described in Materials and Methods.

[c]Values are the mean ± S.E. from two determinations. N.D. = not detected.

[d]Keto aldehyde was detected as the keto aldehyde-bisulfite adduct.

*Significantly different ($p \leq 0.05$) from control value.

Table 4 shows the results of the phenolic compounds on NNK metabolism by ß-naphthoflavone-induced microsomes from hamster liver. (+)-Catechin had no significant effect. Ellagic acid, esculetin and propyl gallate inhibited both methyl and α-methylene hydroxylation although the amounts of metabolites were not significantly different from control values. There was a statistically significant inhibition of N-oxidation and an increase in carbonyl reduction by these three phenols. (-)Esculin inhibited NNAL formation but increased the formation of diol.

Table 4. Effects of phenolic compounds (0.4 μmole) on the metabolism of NNK by β-naphthoflavone-induced hamster liver microsomes.

METABOLITE[a] ρmol/mg/min[c]	TREATMENT[b]					
	Control	(+)-Catechin	Ellagic Acid	Esculetin	(-)Esculin	Propyl gallate
Hydroxy acid (c)	1.0 ± 0.4	0.4 ± 0.1	0.1 ± 0.1	N.D.	0.6 ± 0.1	0.2 ± 0.2
Keto acid (c)	12.9 ± 3.3	19.1 ± 0.2	2.8 ± 0.5	4.6 ± 1.2	25.8 ± 0.4	9.7 ± 4.4
Diol (c)	4.8 ± 1.2	7.9 ± 0.6	N.D.*	0.8 ± 0.8	9.8 ± 0.8*	N.D.*
Keto alcohol (c)	38.2 ± 1.8	35.9 ± 0.6	19.0 ± 0.4*	12.0 ± 0.4*	39.4 ± 0.5	30.9 ± 2.2
Keto aldehyde[d] (c)	1.4 ± 0	0.6 ± 0.2	N.D.*	N.D.*	1.1 ± 0.05	N.D.*
NNAL-N-oxide (b)	24.2 ± 1.1	24.0 ± 3.5	5.3 ± 0.1*	5.1 ± 1.0*	18.4 ± 1.2	9.0 ± 0.05*
NNK-N-oxide (b)	13.2 ± 0.2	9.4 ± 0.1	3.5 ± 0.2*	3.2 ± 0.05*	11.8 ± 0.1	5.8 ± 0.8*
NNAL (a)	25.6 ± 0.3	23.9 ± 1.2	41.2 ± 1.1*	62.3 ± 6.2*	12.2 ± 0.2*	31.1 ± 0.4*
Total (b)	37.3 ± 1.4	33.3 ± 3.7	8.8 ± 0.3*	8.4 ± 1.1*	30.2 ± 1.4	14.7 ± 0.9*
Total (c)	58.2 ± 3.1	64.0 ± 0.2	22.0 ± 0.01*	17.4 ± 0.9*	76.9 ± 0.7*	40.9 ± 2.0*
% unmetabolized NNK + NNAL	26.1 ± 0.5	23.8 ± 0.2	76.4 ± 0.2*	79.3 ± 4.3*	10.9 ± 0.2*	56.6 ± 0.3*

[a]Refer to Figure 7 for names of metabolites and metabolic pathways (given in parentheses).

[b]In vitro incubations were carried out as described in Materials and Methods.

[c]Values are the mean ± S.E. from two determinations. N.D. = not detected.

[d]Keto aldehyde was detected as the keto aldehyde-bisulfite adduct.

*Significantly different ($p \leq 0.05$) from control value.

DISCUSSION

In this study, hamsters were treated with multiple doses of phenobarbital or ß-naphthoflavone according to Paolini et al., 1991 who showed that this treatment increases total P-450 and its activities. These two inducers were selected because rat P-450 2B orthologue induced by phenobarbital has been shown to metabolize NNK. ß-naphthoflavone induces P-450 1A and the human orthologues of P-450 1A can activate NNK to mutagenic intermediates [Crespi et al., 1991]. In this study, we observed an induction of the hamster orthologues of P-450 1A2 or P-450 2B1 and 1B2 (Figure 1). These proteins were recognized by anti-rabbit antibodies suggesting a similarity between the rabbit and hamster P-450 orthologues. ß-naphthoflavone belongs to the "3-methylcholanthrene-type" of inducers known to increase the transcription of P-450 1A1 and P-450 1A2 in rat liver [Okay, 1990]. Sagami et al., observed a high amino acid sequence homology between the rat P-450 1A2 and one of the two major forms of cytochrome P-450 (P-450 MCI) isolated from liver of 3-methylcholanthrene-treated hamsters [Sagami et al., 1991].

The orthologue P-450 1A1 protein would present at a very low level in hamster liver. Our metabolism studies suggest that the orthologue P-450 1A2 mediated the N-oxidation of NNK in hamster liver (Tables 2 and 4). The N-oxide metabolite produced by this metabolic pathway is more polar and far less carcinogenic than NNK [Castonguay et al., 1983]. Our results with liver P-450 of phenobarbital-treated hamsters are in line with those of Ryan who observed an increase in the expression of two anti-2B immunoreactive liver proteins by phenobarbital treatment [Ryan, 1993]. In rat liver, both P-450 2B1 and P-450 2B2 are induced by phenobarbital [Wolf et al., 1984]. The hamster orthologues 2B1 and 2B2 catalyzed the N-oxidation of NNK. Phenobarbital treatment of rat also increased N-oxidation of NNK [Guo et al., 1992].

We observed that both inducers significantly increased the mutagenicity of NNK activated by hamster liver microsomes (Figure 6). This increase was paralleled by an enhancement in α-carbon hydroxylation of NNK and a lower recovery of NNK + NNAL with both inducers. It was previously observed that the induction of α-carbon hydroxylation in hamster lung tissues by phenobarbital

was negligible [Charest et al., 1989]. As shown in Tables 2-4, methyl hydroxylation leading to the keto alcohol was far more important than α-methylene hydroxylation. Using two precursors of NNK-derived electrophiles, Hecht et al. [Hecht et al., 1983] have suggested that pyridyloxobutylation and methylation of bacterial DNA in TA1535 strain was equally mutagenic. It has also been observed that methyl hydroxylation was higher than α-methylene hydroxylation in A/J mouse liver [Peterson et al., 1991a]. It is important to note that methylation of bacterial DNA might still be more abundant than pyridyloxobutylation since methylating agents are alkylating DNA more efficiently than pyridyloxobutylating agents [Hecht et al., 1988].

In this study, P-450 induction in hamster liver increased the mutagenic potential of NNK (Figure 6). These results are in line with those of Alaoui-Jamali et al., who showed that treating hamsters with polychlorinated biphenyls increases the genotoxicity of NNK [Alaoui-Jamali et al., 1988]. The five phenolic compounds selected have dietary or medicinal relevance. Ellagic acid is a component of ellagitannins found in various soft fruits and nuts [Haslam, 1981].

(+)-Catechin, a polyphenol, is found in peels of apples and tea leaves [Chen, 1983]. Esculetin and the related (-)esculin are products of certain Nigerian medicinal plants (Uwaifo, 1984]. Esculetin is reported to have antilipooxygenase and anticyclooxygenase activity [Gochwendt et al., 1984]. Propyl gallate is a phenolic compound with antioxidant properties [Fund et al., 1985].

We observed that the inhibition of mutagenicity and metabolism of NNK by polyphenolic compounds were correlated to some extent. With the ß-naphthoflavone induced system, the three best antimutagenic phenols, ellagic acid, esculetin and propyl gallate, significantly inhibited the overall metabolism of NNK + NNAL (Table 4). These three phenols inhibited the formation of the keto aldehyde by α-methylene hydroxylation. However, only two of these phenols, ellagic acid and esculetin, inhibited the formation of the keto alcohol (methyl hydroxylation pathway). It is intriguing that these three phenols also inhibited the N-oxidation pathway (Figure 7, pathway b) which is considered to be involved in detoxication of NNK and NNAL. Using the phenobarbital induced microsomes, we observed that ellagic acid and esculetin inhibited the mutagenicity

and overall metabolism of NNK + NNAL (Table 3). In contrast, the other antimutagenic phenol, propyl gallate, did not inhibit the metabolism of NNK. With the control microsomes, we observed a good correlation between the antimutagenicity of ellagic acid, propyl gallate, esculetin and (-) esculin and the inhibition of α-methylene hydroxylation (Table 2). We also noted an inhibition of NNK N-oxidation with three of the phenols. We conclude from this comparative study that inhibition of metabolism is contributing to the antimutagenicity of phenolic compounds, although some other unknown factors may also be involved. Guo et al., have demonstrated that purified rat P-450 2B1 was catalyzing the α-carbon hydroxylation and pyridine N-oxidation of NNK [Guo et al., 1991]. In our study, both pathways were generally inhibited to a similar extent, supporting their observation.

Daily consumption of phenolic compounds could reach 1 g [Kuehnau, 1976] and play a significant role in the dietary protection against environmental mutagens. Chemoprevention of mutagenesis and carcinogenesis is reported to occur by: inhibition of enzymes involved in the activation of a procarcinogen to mutagenic and

carcinogenic intermediates [Newmark, 1984]; enhancement of detoxification pathways [Wattenberg, 1985] and/or scavenging of the reactive intermediates to prevent their reaction with DNA [Newmark, 1984]. From this study, the inhibition of the enzymes involved in the activation of NNK associated with a reduction of DNA damage is a likely mechanism of chemoprevention of NNK-carcinogenesis. One of the best antimutagens observed in this study, ellagic acid, has been shown to be an efficient inhibitor of NNK-induced tumorigenesis [Pepin et al., 1990]. Castonguay et al., also demonstrated that ellagic acid inhibited α-carbon hydroxylation of NNK and formation of methylated guanines [Castonguay et al., 1989]. O^6-Guanine is an important determinant of NNK tumorigenesis [Peterson et al., 1991b].

In summary, our results demonstrate that the relative antimutagenic efficacies of polyphenolic compounds was dependant on the enzyme induction treatment. While ellagic acid was the most efficient inhibitor with control microsomes, propyl gallate was the most efficient inhibitor with phenobarbital and ß-naphthoflavone induced microsomes. This suggests that phenolic compounds have a selective affinity for specific isozymes of cytochrome P-450.

ACKNOWLEDGMENTS

This study was supported in part by a grant from the National Cancer Institute of Canada (Toronto). We thank Dr. Bruce Ames, Univ. Calif., Berkeley for providing the strain 1535 of *Salmonella typhimurium* and Dr. Richard M. Philpot, Univ. North Carolina, for providing antibodies and facilities for Western analyses.

REFERENCES

Adesnik M, Atchison M (1985): Genes for cytochrome P-450 and their regulation. CRC Crit Rev Biochem 19:247-305.

Alaoui-Jamali MA, Castonguay A, Schuller HM (1988): *In vitro* genotoxicity of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone activated by hamster maternal and fetal tissues. Carcinogenesis 9:2319-2324.

Brunnemann KD, Cox JE, Hoffmann D (1992): Analysis of tobacco-specific N-nitrosamines in indoor air Carcinogenesis, 13:2415-2418.

Burton HR, Childs GH, Anderson RA, Fleming PD (1989): Changes in chemical composition of Burley tobacco during senescence and curing. 3. Tobacco-specific nitrosamines. J Agric Food Chem 37:426-430.

Castonguay A, Lin D, Stoner GD, Radok P, Furuya K, Hecht SS, Schut HAG, Klaunig JE (1983): Comparative carcinogenicity in A/J mice and metabolism by cultured mouse peripheral lung of N′-nitrosonornicotine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and their analogues. Cancer Res 43:1223-1229.

Castonguay A, Allaire L, Charest M, Rossignol G, Boutet M (1989): Metabolism of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone by hamster respiratory tissues cultured with ellagic acid. Cancer Lett 46:93-105.

Charest M, Rossignol G, Castonguay A (1989): *In vitro* and in vivo modulation of the bioactivation of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone in hamster lung tissues. Chem-Biol Interact 71: 265-278.

Chen ZK (1983): The chemistry and biochemistry of tea. Chinese Tea Research Institute Press Inc., Hangzhau, China.

Crespi CL, Penman BW, Gelboin HV, Gonzalez FJ (1991): A tobacco smoke derived nitrosamine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, is activated by multiple human cytochrome P-450s including polymorphic human cytochrome P-450 2D6. Carcinogenesis 12:1197-1201.

Devereux TR, Anderson MW, Belinsky SA (1988): Factors regulating activation and DNA alkylation by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and nitrosodimethylamine in rat lung and isolated lung cells and the relationship to carcinogenicity. Cancer Res 48:4215-4221.

Domin BA, Serabjit-Singh CJ, Philpot RM (1984): Quantitation of rabbit cytochrome P-450, form 2, in microsomal preparations bound directly to nitrocellulose paper using a modified peroxidase-immunostaining technique. Anal Biochem 136:390-396.

Fischer S, Spiegelhalder B, Eisenbarth J, Preussmann R (1990a): Investigations on the origin of tobacco-specific nitrosamines in mainstream smoke of cigarettes. Carcinogenesis, 11:723-730.

Fischer S, Spiegelhalder B, Preussmann R (1990b): Tobacco-specific nitrosamines in European and USA cigarettes. Arch Geschwulstforsch 3:169-177.

Fund DY, Liu LL, Gailani MB (1985): Effect of phenolic antioxidants on microbial growth. CRC Crit Rev Microbiol 12:153-183.

Gochwendt M, Horn F, Kittstein W, Furstenberger G, Besemfelder E, Marks F (1984): Calcium and phospholipid-dependent protein kinase activity in mouse epidermis cytosol: stimulation by complete and incomplete tumor promoters and inhibition by various compounds. Biochem Biophys Res Commun 124:63-68.

Guo Z, Smith TJ, Ishizaki H, Yang CS (1991): Metabolism of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) by cytochrome P-450 IIB1 in a reconstituted system. Carcinogenesis 12:2277-2282.

Guo Z, Smith TJ, Thomas PE, Yang CS (1992): Metabolism of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone by inducible and constitutive cytochrome P-450 enzymes in rats. Arch Biochem Biophys 298:279-286.

Guttenplan JB (1980): Enhanced mutagenic activities of N-nitroso compounds in weakly acidic media. Carcinogenesis 1:439-444.

Haslam E (1981): In: The Biochemistry of Plants, Vol 7, pp 527-533. Ed.: Conn EV Academic Press, New York, NY.

Hecht SS, Young R, Chen CB (1980): Metabolism in the F344 rat of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, a tobacco-specific carcinogen. Cancer Res 40:4144-4150.

Hecht SS, Lin D, Chen CB (1981): Comprehensive analysis of urinary metabolites of N′-nitrosonornicotine. Carcinogenesis 2:833-838.

Hecht SS, Lin D, Castonguay A (1983): Effects of α-deuterium substitution on the mutagenicity of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). Carcinogenesis 4:305-310.

Hecht SS, Spratt TE, Trushin N (1988): Evidence for 4-(3-pyridyl)-4-oxobutylation of DNA in F344 rats treated with the tobacco-specific nitrosamines 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and N′-nitrosonornicotine. Carcinogenesis 9:161-165.

Hecht SS, Hoffmann D (1988): Tobacco-specific nitrosamines, an important group of carcinogens in tobacco and tobacco smoke. Carcinogenesis 9:875-884.

Hecht SS, Hoffmann D (1989): The relevance of tobacco specific nitrosamines to human cancer. Cancer Surv 8:273-294.

Hirayama T (1984): Lung cancer in Japan: effects of nutrition and passive smoking. In: Lung Cancer: causes and prevention, pp 175-195. Eds.: Mizell M, Correa P Verlag Chemie International Inc., Deerfield Beach, FL.

Hong J-Y, Ding X, Smith TJ, Coon MJ, Yang CS (1992): Metabolism of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, a tobacco-specific carcinogen, by rabbit nasal microsomes and cytochrome P-450s NMa and NMb. Carcinogenesis 13:2141-2144.

Jorquera R, Castonguay A, Schuller HM (1992): Effects of pregnancy and ethanol on the metabolism of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone by hamster liver and lung microsomes. Drug Metab Dispos 20:510-517.

Kuehnau J (1976): The flavonoids. A class of semi-essential food components. Their role in human nutrition. World Rev Nutr Diet 24:117-191.

Lord HL , Snieckus VA, Josephy PD (1989): Re-evaluation of the effect of ellagic acid on dimethylnitrosamine mutagenicity. Mutagenesis, 4:453-455.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951): Protein measurement with the Folin phenol reagent. J Biol Chem 192:265-275.

Mandal S, Ahuja A, Shivapurkar NM, Cheng SJ, Groopman JD, Stoner GD. (1987): Inhibition of aflatoxin B_1 mutagenesis in *Salmonella typhimurium* and DNA damage in cultured rat and human tracheobronchial tissues by ellagic acid. Carcinogenesis 8:1651-1656.

Maron DM, Ames BN (1983): Revised methods for the *Salmonella* mutagenicity test. Mutat Res 113:173-215.

Murphy SE, Palomino A, Hecht SS, Hoffmann D (1990): Dose-response study of DNA and hemoglobin adduct formation by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone in F344 rats. Cancer Res 50:5446-5452.

Nagabhushan M, Bhide SV (1988): Antimutagenicity of catechin against environmental mutagens. Mutagenesis 3:293-296.

Newmark HL (1984): A hypothesis for dietary components as blocking agents of chemical carcinogenesis: plant phenolics and pyrrole pigments. Nutr Cancer 6:58-70.

Paolini M, Sapigni E, Hrelia P, Scotti M, Morotti M, Cantelli-Forti G (1991): Wide spectrum detection of precarcinogens in short-term bioassays by simultaneous superinduction of multiple forms of cytochrome P-450 isoenzymes. Carcinogenesis 12:759-766.

Pepin P, Rossignol G, Castonguay A (1990): Inhibition of NNK-induced lung tumorigenesis in A/J mice by ellagic acid and butylated hydroxyanisole. Cancer J 3:266-273.

Peterson LA, Mathew R, Hecht SS (1991a): Quantitation of microsomal α-hydroxylation of the tobacco-specific nitrosamine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone. Carcinogenesis 12:2069-2072.

Peterson LA, Hecht SS (1991b): O^6-Methylguanine is a critical determinant of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone tumorigenesis in A/J mouse lung. Cancer Res 51:5557-5564.

Ryan R, Ph.D. (1993): Thesis, University of North Carolina.

Sagami I, Ohmachi T, Fujii H, Kikuchi H and Watanabe M (1991): Hamster cytochrome P-450 IA gene family, P-450 IA1 and P-450 IA2 in lung and liver: cDNA cloning and sequence analysis. J Biochem 110:641-647.

Serbjit-Singh C-J, Wolf CR, and Philpot RM (1979): The rabbit pulmonary monoxygenase system: Immunochemical and biochemical characterization of enzyme components. J Biol Chem 254:9901-9907.

Smith TJ, Guo Z, Thomas PE, Chung FL, Morse MA, Elkind K, Yang CS (1990): Metabolism of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone in mouse lung microsomes and its inhibition by isothiocyanates. Cancer Res 50:6817-6822.

Smith TJ, Guo Z, Hong JY, Ning SM, Thomas PE, Yang CS (1992): Kinetics and enzyme involvement in the metabolism of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone in microsomes of rat lung and nasal mucosa. Carcinogenesis 13:1409-1414.

Teel RW (1992): Effects of different inducers of cytochrome P-450 on mutagenesis of the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in *Salmonella typhimurium* TA1535. Anticancer Res 12:1287-1290.

Teel RW, Castonguay A (1992): Antimutagenic effects of polyphenolic compounds. Cancer Lett 66:107-113.

Tynes RE, Philpot RM (1987): Tissue- and species-dependent expression of multiple forms of mammalian microsomal flavin-containing monooxygenase. J Pharmacol Exp Ther 31:569-574.

Uwaifo AD (1984): The mutagenicities of seven coumarin derivatives and a furan derivative isolated from three medicinal plants. J Toxicol Environ Health 13:521-530.

Wattenberg LW (1985): Chemoprevention of cancer. Cancer Res 45:1-8.

Wolf CR, Moll E, Friedberg T, Oesch F, Buchmann A, Kuhlmann WE, Kunz HW (1984): Characterization, localization and regulation of a novel phenobarbital-inducible form of cytochrome P-450, compared with three further P-450 isoenzymes, NADPH P-450 reductase, glutathione transferase and microsomal epoxide hydrolase. Carcinogenesis 5:993-1001.

Wood AW, Huang MT, Chang RL, Newmark HL, Lehr RE, Yagi H, Sayer JM, Jerina DM, Conney AH (1982): Inhibition of the mutagenicity of bay-region diol epoxides of polycyclic aromatic hydrocarbons by naturally occurring plant phenols: exceptional activity of ellagic acid. Proc Natl Acad Sci 79:5513-5517.

Yamazaki H, Inui Y, Yun CH, Guengerich FP, Shimada T (1992): Cytochrome P-450 2E1 and 2A6 enzymes as major catalysts for metabolic activation of N-nitrosodialkylamines and tobacco-related nitrosamines in human liver microsomes. Carcinogenesis 13:1789-1794.

Yang CS, Brady JF, Hong J-Y (1992): Dietary effects on cytochrome P-450, xenobiotic metabolism, and toxicity. FASEB J 6: 737-744.

CHAPTER IV

EFFECTS OF COMPOUNDS OF PLANT ORIGIN ON THE MUTAGENICITY AND METABOLISM OF THE TOBACCO-SPECIFIC NITROSAMINE NNK

Cecil H. Miller[1], Solomon M. Hamilton[2], and Robert W. Teel[3]

[1]Graduate student that conducted the experiments, collected the data and helped prepare the manuscript

[2]Graduate student that was being trained and helped by collecting HPLC samples and running them in the scintillation counter

[3]Student's research committee chairman who supervised the research and helped prepare the manuscript

Phytotherapy (1994) in press

EFFECTS OF COMPOUNDS OF PLANT ORIGIN ON THE MUTAGENICITY AND METABOLISM OF THE TOBACCO-SPECIFIC NITROSAMINE NNK

C.H. Miller, S.M. Hamilton, and R.W. Teel

Department of Physiology and Pharmacology,
School of Medicine, Loma Linda University,
Loma Linda, CA 92350

Key words: Chemoprevention; mutagenicity; metabolism; NNK; phytochemicals

Correspondence: R.W. Teel, Ph.D.
Department of Physiology and Pharmacology
School of Medicine, Loma Linda University
Loma Linda, CA 92350 (U.S.A.)

SUMMARY

We have investigated the effects of five phytochemicals on the microsomal-dependent mutagenicity and metabolism of the tobacco-specific nitrosamine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). Two compounds, d-limonene and silymarin, had no effect on NNK-induced mutagenesis in *Salmonella typhimurium* TA1535 over the concentration range of 0.1 - 0.4 μmoles/plate. Diallyl sulfide was weakly antimutagenic at a concentration of 0.4 μmoles/plate. Both capsaicin and tannic acid showed a dose-dependent inhibition of mutagenesis in TA1535. Metabolism studies using [^{3}H]NNK indicated that the effects of the phytochemicals on NNK-induced mutagenesis did not always correlate with the effects on NNK metabolism. α-Carbon hydroxylation reactions are considered the most significant pathways involved in the metabolic activation of NNK to mutagenic and carcinogenic species. d-Limonene and silymarin (0.4 μmoles) had the least inhibitory effect on the total α-carbon hydroxylation reactions, 19% and 28%. Capsaicin and diallyl sulfide inhibited these pathways by 74% and 70%. Tannic

acid, the most potent phytochemical tested in this study, inhibited total α-carbon hydroxylation pathways by 99%.

INTRODUCTION

The nicotine-derived nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) is a product of the nitrosation of nicotine and its derivatives during tobacco processing. It is reported to produce tumors in several animal species (Hecht and Hoffman, 1988; Rivenson et al., 1988; Hecht et al., 1989). NNK requires cytochrome P450-dependent metabolic activation to exhibit its mutagenicity and carcinogenicity (Hecht et al., 1983, Hecht and Hoffmann, 1988). The metabolism of NNK includes carbonyl reduction, N-oxidation of the pyridine ring and α-carbon hydroxylation reactions. The latter can result in the methylation and pyridyloxobutylation of purine bases of DNA (Murphy et al., 1990). Several cytochrome P450 isozymes have been associated with the metabolic activation of NNK including P450 2B1, 2B2 and P450 1A1 (Devereux et al., 1988; Smith et al., 1990; Guo et al., 1991).

Because epidemiological studies have implicated the use of tobacco products with human cancer (Hecht and Hoffmann, 1989), it is important to identify chemical compounds of natural or synthetic origin that modulate the metabolism of NNK and hence its

mutagenicity and carcinogenicity. Phytochemicals with potential chemopreventive properties, are widely distributed and are present in various foods humans normally eat (Haslam, 1981). There are several reports describing the antimutagenic and anticarcinogenic properties of plant phenolic compounds (Imaida et al., 1992; Kuehnau, 1976; Liu and Castonguay, 1991; Mandal et al., 1987; Nagabhushan and Bhide, 1988; Newmark, 1984; Wood et al., 1982).

In the interest of identifying potential chemopreventive compounds, we compared the effects of five phytochemicals on the mutagenicity and metabolism of NNK when activated by liver microsomes from male Syrian golden hamsters.

MATERIALS AND METHODS

Chemicals

[5-^{3}H] NNK (1.51 Ci/mmole) and unlabeled NNK (purity >97%) were purchased from Chemsyn Science Laboratory (Lenexa, KS). The synthesis of NNK metabolites used as reference standards for HPLC analysis has been reported (Hecht et al., 1981). $NADP^+$, glucose-6-phosphate, glucose-6-phosphate dehydrogenase and dimethylsulfoxide (DMSO), capsaicin, diallyl sulfide, d-limonene, silymarin and tannic acid were obtained from Sigma Chemical Co. (St. Louis, MO). Bactoagar was purchased from VWR Scientific (Cerritos, CA) and nutrient broth No. 1 was purchased from Oxoid Co. (Basingstoke, Hants, UK).

Preparation of microsomes

Male Syrian golden hamsters, 150-160 g (Charles River Laboratory, Wilmington, ME) were given water and Purina rodent laboratory chow ad libitum for five days prior to time of sacrifice. Animals were sacrificed by cervical dislocation and the livers were removed aseptically and homogenized in two volumes of 0.25 M

Tris-HCl (pH 7.4) containing 0.15 M sucrose and 1mM EDTA. Microsomes were obtained by differential centrifugation of the homogenate (Maron and Ames, 1983). The microsome pellets were resuspended in Tris-HCl:sucrose:EDTA buffer and microsome fractions from four hamsters in each treatment group were pooled. Aliquots from pooled samples were stored at -80°C. Protein content was determined (Lowry et al., 1951).

Assay of NNK-induced mutagenesis

The effect of nontoxic concentrations of the phytochemicals (Teel, 1993) on hamster liver microsome-mediated mutagenicity of NNK was performed as follows. Sterile tubes containing 1 mg liver microsomal protein, 80 mM unlabeled NNK in 100 μl saline, cofactor buffer pH 6.8 (Guttenplan, 1980), 0.15 ml of a five h culture of *Salmonella typhimurium* strain TA1535 (a gift from B. Ames, Univ. Calif., Berkeley) and 0, 0.1, 0.2, or 0.4 μmoles capsaicin, diallyl sulfide, d-limonene, silymarin and tannic acid in DMSO in a final volume of one ml were incubated in triplicate in a shaking water bath at 37°C for 30 min. The volume of DMSO was kept uniform in

all incubations to correct for its reported inhibitory effect (Teel, 1992b). Two ml of top agar was added to each tube following the 30 min incubation and the contents were poured onto minimal essential agar plates (Maron and Ames, 1983). The plates were incubated for 48 hrs at 37°C after which the number of histidine-independent (His^+) colonies were counted. Results were expressed as the mean number of NNK-induced His^+ revertant colonies/plate. Spontaneous revertant colonies (9-12/plate) were subtracted.

Assay of NNK Metabolism

The effect of phytochemicals on the microsome-mediated metabolism of NNK was performed as a modification of a procedure described by Smith et al., 1990. The incubation mixture contained 5 mM glucose-6-phosphate, 1.5 units glucose-6-phosphate dehydrogenase, 1 mM $NADP^+$, 1 mM EDTA, 3 mM $MgCl_2$, 12.8 μCi [5-^{3}H]NNK, 0.4 μmole phytochemical in DMSO and 5 mM sodium bisulfite in a final volume of 1 ml. Following incubation at 37°C for 10 min, the reaction was started by the addition of 1 mg microsomal protein. Blanks were incubated with boiled microsomes. The

reaction mixture was incubated for 30 min in a shaking water bath at 37°C and stopped by the addition of 200 µl saturated barium hydroxide and 200 µl 25% zinc sulfate. The precipitated samples were centrifuged at 14,000 xg for 20 min and filtered through 0.45 µm cameo filters (MSI, Westboro, MA). One hundred µl of the filtrate and 7 µl of NNK metabolite standards were coinjected onto a reversed-phase HPLC system using a 10 µm Bondapak C18 column (3.9 mM x 300 mM, Waters, Milford, MA). NNK and its metabolites were eluted with sodium acetate buffer (pH 6) and methanol (Peterson et al., 1991). Sodium bisulfite (one mM) was in the elution buffer to trap the unstable keto aldehyde product. The eluate was monitored at 254 nm and one ml fractions were collected. Five ml scintillation cocktail (Scintiverse LC, Fisher Scientific, Tustin, CA) was added to each fraction and the radioactivity was determined by liquid scintillation spectroscopy (Model LS5801 counter, Beckman Instruments, Berkeley, CA). Recovery of total radioactivity during HPLC analysis was > 70%. Analysis of the radioactivity was performed using a Beckman Datagraph Program in the DU Data

leader software package on an IBM PS/2 model 50 computer and expressed as pmoles/mg microsome protein/min.

Statistical Analysis

Data were analyzed by the student's unpaired t-test using the MacIntosh Stat-View software program. Statistical significance was defined at $p < 0.05$ compared with control values.

RESULTS

Mutagenesis of NNK

The effects of capsaicin, diallyl sulfide, d-limonene, silymarin and tannic acid at concentrations of 0, 0.1, 0.2 and 0.4 µmoles/plate on the mutagenicity of NNK in *S. typhimurium* TA1535 by noninduced hamster liver microsomes are shown in Figure 1A-E. Capsaicin and tannic acid significantly inhibited the mutagenicity of NNK in TA1535 at concentrations of 0.1, 0.2 and 0.4 µmoles/plate. Diallyl sulfide expressed inhibition at a concentration of 0.4 µmoles/plate. Neither silymarin or d-limonene inhibited the mutagenicity of NNK in TA1535 at the concentrations tested.

Metabolism of NNK

The pathways in the metabolism of NNK are shown in Figure 2. NNAL is a product of carbonyl reduction of NNK (pathway a). N-oxidation of the pyridine ring of NNK and NNAL yields the N-oxides, NNK-N-oxide and NNAL-N-oxide (pathway b). Primary α-carbon hydroxylation reactions produce keto alcohol and keto aldehyde, indicators of methyl and methylene hydroxylation

Figure 1. Effect of different concentrations of five phytochemicals on the mutagenesis of NNK in *Salmonella typhimurium* TA1535 mediated by hamster liver microsomes. Procedural details for performing the experiments are given in Materials and Methods. Values are means μ S.E.M. of triplicate samples. Asterisks indicate statistically significant difference from control (0 μ mole/plate). $p < 0.05$.

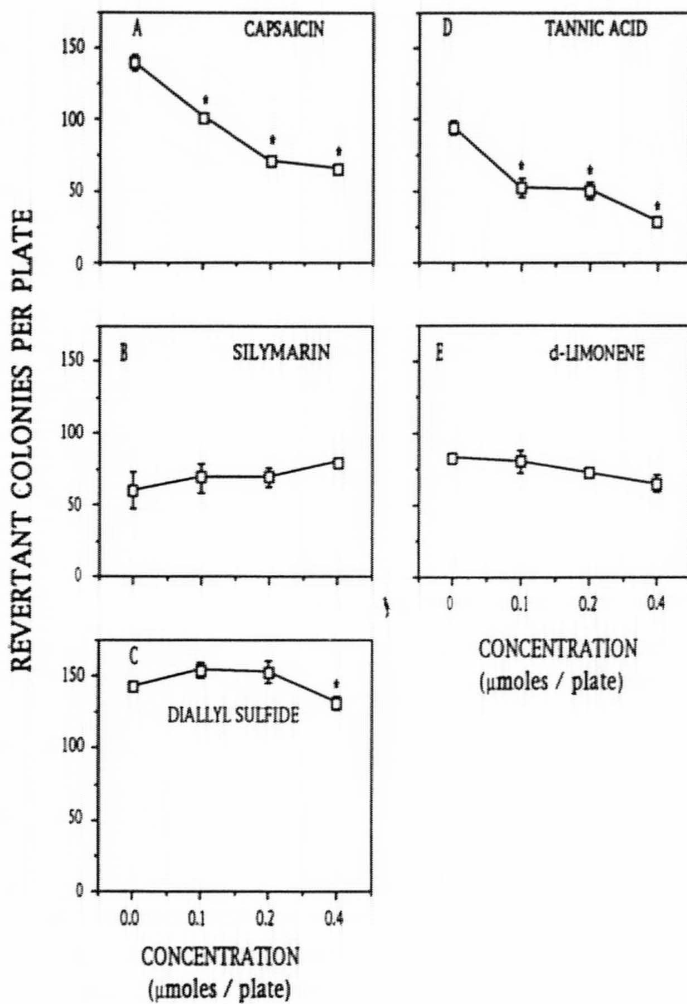
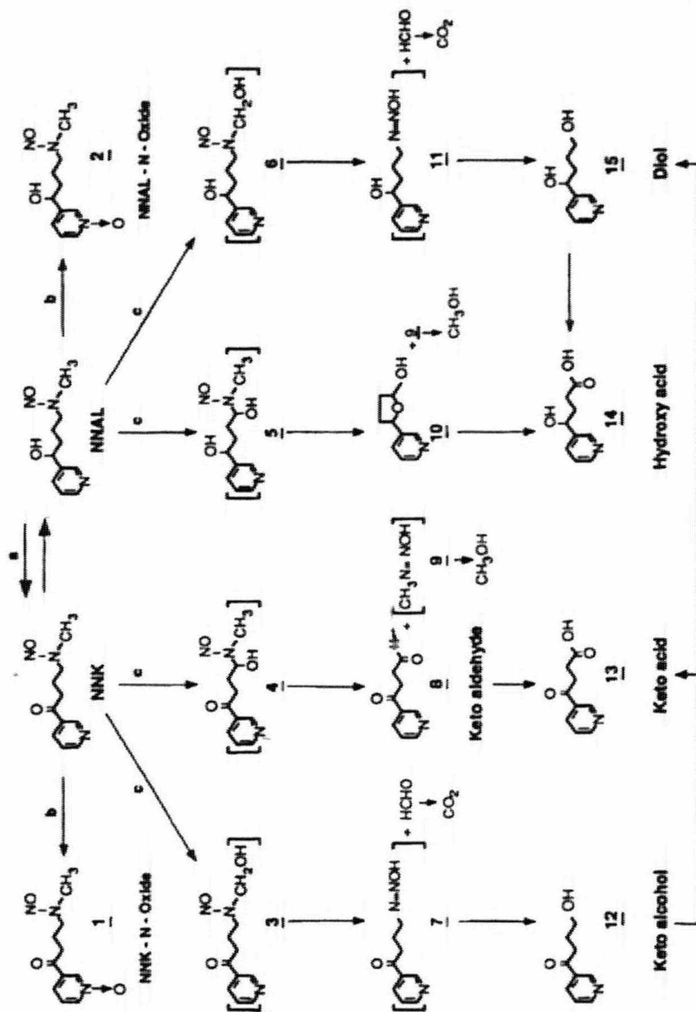

REVERTANT COLONIES PER PLATE
A
CAPSAICIN
D
TANNIC ACID
B
SILYMARIN
E
d-LIMONENE
C
DIALLYL SULFIDE
150
100
50
0
0.0
0.1
0.2
0.4
CONCENTRATION
(μmoles / plate)

Figure 2: Metabolic pathways of NNK: carbonal reduction (pathway a), pyridine N-oxidation (pathway b), and α-carbon hydroxylation (pathway c).
Metabolites: NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol;
NNK-N-oxide, 4-(methylnitrosamino)-1-(3-pyridyl-N-oxide)-1-butanone;
NNAL-N-oxide, 4-(methylnitrosamino)-1-(3-pyridyl-N-oxide)-1-butanol;
keto alchol, 4-oxo-4-(3-pyridyl)-1-butanol;
hydroxy acid, 4-hydroxy-4-(3-pyridyl) butyric acid;
keto aldehyde, 4-oxo-4-(3-pyridyl) butanal;
keto acid, 4-oxo-4-(3-pyridyl) butyric acid;
diol, 4-hydroxy-1-(3-pyridyl)-1-butanol.
The structures in brackets are hypothetical intermediates.
(Adapted from Jorquera et al., 1992; Hecht et al., 1980).



NNK - N - Oxide
NNK
NNAL
NNAL - N - Oxide
Keto aldehyde
Keto alcohol
Keto acid
Hydroxy acid
Diol
HCHO
CO2
CH3OH

respectively (pathways c). The inclusion of 1 mM sodium bisulfite in the incubation medium and HPLC solvent buffers traps the keto aldehyde as a keto aldehyde-bisulfite adduct (Peterson et al., 1991). The subsequent oxidation/reduction of keto alcohol and keto aldehyde yields the secondary α-carbon hydroxylation metabolites keto acid, hydroxy acid and diol.

Table 1 shows that in the control sample (no phytochemicals), the two major metabolites of NNK in the presence of hamster liver microsomes were keto alcohol and NNAL. Carbonyl reduction (pathway a, Figure 2) accounted for 47% of the total metabolism of NNK by these microsomes. Primary and secondary α-carbon hydroxylation (pathway c, Figure 2) accounted for 41% and N-oxidation of the pyridine ring (pathway b; Figure 2) 12%. The unstable keto aldehyde was trapped as the keto aldehyde bisulfite adduct. Data in Table 1 show that capsaicin and tannic acid significantly inhibited the formation of each metabolite of NNK. Diallyl sulfide inhibited the formation of all metabolites except NNAL. d-Limonene inhibited the formation of keto alcohol but enhanced the formation of keto acid, keto aldehyde and NNAL. Silymarin inhibited

Table 1. Effects of phytochemicals on the metabolism of NNK by hamster liver microsomes.

METABOLITE[a] pmol/mg/min[c]	TREATMENT[b] Control	Capsaicin	d-Limonene	Diallyl Sulfide	Silymarin	Tannic Acid
Hydroxy acid (c)	N.D.	N.D.	N.D.	N.D.	0.26 ± 0.03*	N.D.
Keto acid (c)	0.4 ± 0.02	0.13 ± 0.04*	0.54 ± 0.03*	0.09 ± 0.02*	0.48 ± 0.08	N.D.*
Diol (c)	2.15 ± 0.04	0.59 ± 0.08*	2.61 ± 0.21	0.92 ± 0.04*	1.68 ± 0.12*	0.08 ± 0.01*
Keto alcohol (c)	18.44 ± 0.08	4.85 ± 0.26*	13.41 ± 0.88*	5.39 ± 1.17*	12.94 ± 0.23	0.03 ± 0.01*
Keto aldehyde[d] (c)	0.32 ± 0.05	N.D.*	0.65 ± 0.08*	0.1 ± 0.03*	N.D.*	N.D.*
NNAL-N-oxide (b)	1.66 ± 0.05	0.91 ± 0.19*	2.25 ± 0.29	0.81 ± 0.12*	2.01 ± 0.23	0.07 ± 0.03*
NNK-N-oxide (b)	4.51 ± 0.07	N.D.*	5.42 ± 0.52	2.98 ± 0.16*	4.18 ± 0.07	N.D.*
NNAL (a)	24.4 ± 0.15	4.06 ± 0.26*	25.63 ± 0.27*	29.37 ± 0.41*	14.08 ± 0.6*	5.56 ± 0.15*
Total (b)	6.17 ± 0.11	0.91 ± 0.19*	7.67 ± 0.69	3.79 ± 0.19*	6.19 ± 0.18	0.07 ± 0.03*
Total (c)	21.31 ± 0.11	5.57 ± 0.24*	17.21 ± 0.57*	6.48 ± 1.11*	15.36 ± 0.2*	0.11 ± 0.01*
% unmetabolized NNK + NNAL	55.87 ± 0.1	83.06 ± 0.46*	55.25 ± 1.01	77.53 ± 0.53*	65.35 ± 0.44*	95.59 ± 0.07*

[a]Refer to Figure 2 for names of metabolites and metabolic pathways (given in parentheses).

[b]In vitro incubations were carried out as described in Materials and Methods.

[c]Values are the mean ± S.E. from two determinations. N.D. = not detected.

[d]Keto aldehyde was detected as the keto aldehyde-bisulfite adduct.

*Significantly different ($p \leq 0.05$) from control value.

the formation of diol, keto aldehyde and NNAL. The effects of each phytochemical on total NNK-reduction, total N-oxidation and total α-carbon hydroxylation are shown in Figure 3. Capsaicin and tannic acid significantly inhibited total activity in all three pathways. Although all five phytochemicals inhibited total α-carbon hydroxylation, capsaicin, diallyl sulfide and tannic acid were the most effective. Tannic acid expressed the most inhibition of N-oxidation and α-carbon hydroxylation pathways of any compound tested.

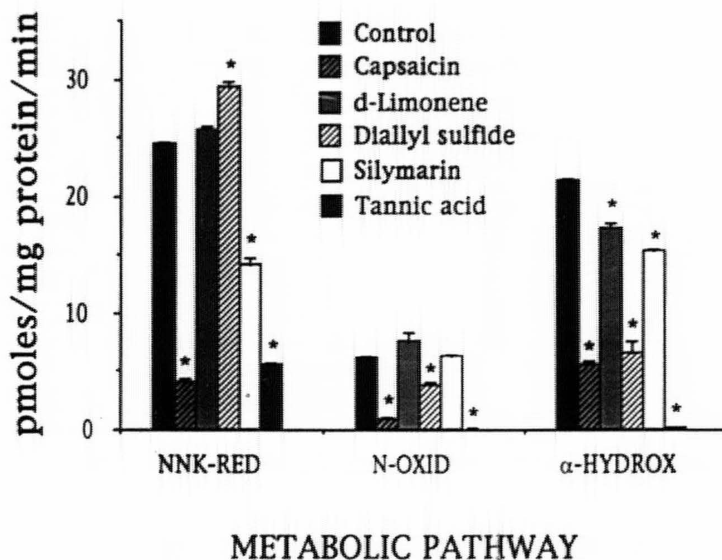


Figure 3: Effects of five phytochemicals on the metabolism of NNK by hamster liver microsones. Total NNK reduction, total pyridine N-oxidation and total a-carbon hydroxylation from Table 1 are shown. Procedural details of incubations and analysis of metabolites are described in Materials and Methods. Values are means +/- SEM of triplicate samples. Asterisks indicate statistically significant difference from control, $p < 0.05$.

DISCUSSION

Chemoprevention of mutagenesis and carcinogenesis is reported to occur by inhibition of enzymes involved in the activation of a procarcinogen to mutagenic and carcinogenic products (Newmark, 1984); enhancement of detoxification pathways (Wattenberg, 1985); and/or scavenging of reactive intermediates that prevents interaction with DNA (Newmark, 1984). Various compounds present in plants, including edible ones, offer promise as chemopreventive agents.

In this study, we report the effects of five phytochemicals on the bioactivation-dependent mutagenesis and metabolism of the tobacco-specific nitrosamine NNK. The metabolism of NNK involves several pathways that include carbonyl reduction of NNK to NNAL (pathway a, Figure 2), N-oxidation of the pyridine ring of NNK and NNAL (pathway b, Figure 2) and α-carbon hydroxylation (pathway c, Figure 2). α-Carbon hydroxylation reactions forming keto aldehyde and a methylating species and keto alcohol and pyridyloxobutylating species are regarded as the pathways critical to the mutagenic and carcinogenic actions of NNK (Murphy et al., 1990).

Capsaicin (8-methyl-N-vanillyl-6-nonenamide) is a pungent, irritating compound of *Capsicum* fruits. These fruits are widely used in the diet (Govindarajan and Sathyanarayana, 1991). Capsaicin inhibited aryl hydrocarbon hydroxylase activity in neonatal epidermal microsomes (Modly et al., 1986) and is reported to inhibit the *in vitro* metabolism of aflatoxin B_1 and the formation of aflatoxin B_1 adducts with calf thymus DNA (Teel, 1991). Capsaicin inhibited rat liver microsomal mixed-function oxidase and epoxide hydratase activity (Teel, 1992a). In this study, capsaicin inhibited hamster liver microsome-mediated mutagenicity of NNK in *S. typhimurium* TA1535 by 59%, which correlated with an inhibition of the metabolism of NNK. Capsaicin inhibited NNK reduction by 82.5%, pyridine N-oxidation by 85% and α-carbon hydroxylation by 74% (Figure 3).

Diallyl sulfide (DAS), a component of garlic, is reported to inhibit a variety of chemically induced tumors in laboratory rodents (Wargovich, 1987; Wargovich et al., 1988; Sparnins et al., 1988; Hadjiolov et al., 1993). These effects have been associated with the inhibition of microsomal monooxygenase activity and/or enhanced

glutathione-S-transferase activity (Sparnins et al., 1988; Brady et al., 1988; Maurya and Singh, 1991; Ludeke et al., 1992). One study found no effect of DAS on rat microsomal mixed function oxidase or epoxide hydratase activity (Teel, 1992a). We found a significant inhibition of pyridine N-oxidation and α-carbon hydroxylation metabolic pathways by DAS and a small but statistically significant enhancement of NNK-reduction. Pyridine N-oxidation was inhibited by 39% and α-carbon hydroxylation by 70%. DAS inhibited the mutagenesis of NNK in *S. typhimurium* TA1535 at the highest nontoxic concentration tested (0.4 μmoles/plate) by 8%.

d-Limonene is a terpenoid compound found in peels of citrus fruits and is reported to inhibit DMBA-induced rat mammary tumors (Elson et al., 1988) and N-nitrosodiethylamine-induced forestomach and pulmonary adenomas in mice (Wattenberg et al., 1989). Steele et al., (1990) found an inhibition of benzo[a]pyrene (BaP)-induced rat tracheal epithelial cell transformation by d-limonene. We found no significant effect of d-limonene on NNK-induced mutagenesis in TA1535 although some inhibition of α-carbon hydroxylation (19%) was observed in this study.

Silymarin, a flavenoid extract from milk thistle (*Silybum marianum*) seeds, is reported to have antioxidant properties (Valenzuela et al., 1986; Bindoli et al., 1977) and inhibited the transformation of rat tracheal cells (Steele et al., 1990). No effect was observed on microsomal mixed function oxidase or epoxide hydratase activity (Teel, 1992a). Silymarin had no effect on NNK-induced mutagenesis in TA1535 in this study, although it inhibited NNK reduction by 42% and α-carbon hydroxylation by 28%.

Tannic acid is present in a variety of plants (Haslam, 1981). It is reported to inhibit the mutagenicity of BaP-7,8-diol-9,10 epoxide in *S. typhimurium* and in Chinese hamster V-79 cells (Huang et al., 1983). It inhibited epidermal monooxygenase activity and binding of BaP and BaP 7,8-dihydrodiol to DNA (Das et al., 1987). It was also shown to inhibit liver S9-mediated mutagenesis, metabolism and DNA binding of BaP (Vance and Teel, 1989). We found that tannic acid inhibited NNK-induced mutagenesis in TA1535 by 69% and inhibited NNK reduction, pyridine N-oxidation and α-carbon hydroxylation by 77%, 99% and 99%, respectively. Tannic acid had

the greatest inhibitory effect on NNK-induced mutagenesis and on the metabolism of NNK of any phytochemical tested in this study.

In summary, five phytochemicals were examined for their effects on the mutagenicity of the tobacco-specific nitrosamine NNK in *S. typhimurium* TA1535 and on the metabolism of NNK by hamster liver microsomes. The effect of the individual phytochemicals on the mutagenicity of NNK did not always correlate with the effect on the metabolism of NNK. Neither silymarin or d-limonene inhibited the mutagenesis of NNK in TA1535 although silymarin inhibited the NNK-reduction and α-carbon hydroxylation pathways of NNK metabolism. d-Limonene showed a small inhibitory effect on α-carbon hydroxylation pathways. DAS exhibited a small inhibitory effect on NNK-induced mutagenesis at the highest nontoxic concentration tested (0.4 μmoles/plate) and at that concentration inhibited both pyridine N-oxidation and α-carbon hydroxylation. Both capsaicin and tannic acid showed a dose-dependent inhibition of NNK-induced mutagenesis in TA1535 and both compounds significantly inhibited NNK reduction, pyridine N-oxidation and α-carbon hydroxylation pathways. The effects of

tannic acid were paramount. These results add support to other studies that suggest the need for a careful evaluation of the chemopreventive properties of phytochemicals and their use as an approach to the prevention of human cancers.

ACKNOWLEDGEMENTS

The assistance of Velma Clem in the preparation of the manuscript is greatly appreciated.

REFERENCES

Bindoli, A., Cavallini, L., and Siliprandi, N. (1977). Inhibitory action of silymarin of lipid peroxide formation in rat liver mitochondria and microsomes. Biochem. Pharmacol. 26, 2405-2409.

Brady, J.F., Li, D., Ishizaki, H., and Yang, C.S. (1988). Effect of diallyl sulfide on rat liver microsomal nitrosamine metabolism and other monooxygenase activities. Cancer Res. 48, 5937-5960.

Das, M., Khan, W.S., Asokan, P., Bickers, D.R., and Mukhtar, H. (1987). Inhibition of polycyclic aromatic hydrocarbons-DNA adduct formation in epidermis and lungs of SENCAR mice by naturally occurring plant phenols. Cancer Res. 47, 767-773.

Elson, C.E., Maltzman, T. H., Boston, J.L., Tanner, M.A., and Gould, M.N. (1988). Anti-carcinogenic activity of D-limonene during the initiation and promotion/progression stages of DMBA-induced rat mammary carcinogenesis. Carcinogenesis 9, 331-332.

Devereux, T.R., Anderson, M.,W. and Belinsky, S.A. (1988): Factors regulating activation and DNA alkylation by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and nitrosodimethylamine in rat lung and isolated lung cells and the relationship to carcinogenicity. Cancer Res. 48, 4215-4221.

Govindarajan, V.S., and Sathyanarayana, M.N. (1991). Capsicum production, technology, chemistry and quality. Part V. Critical Rev. Food Science and Nutrition. 29, 435-474.

Guo, Z., Smith, T.J. Ishizaki, H., and Yang, C.S. (1991). Metabolism of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) by cytochrome P450 IIB1 in a reconstituted system. Carcinogenesis 12, 2277-2282.

Guttenplan, J.B. (1980). Enhanced mutagenic activities of N-nitroso compounds in weakly acidic media. Carcinogenesis 1, 439-444.

Hadjiolov, D., Fernando, R.C., Schmeister, H.H., Wiebler, M., Hadjiolov, N., and Pirajnov, G. (1993). Effect of diallyl sulfide on aristolochic acid-induced forestomach carcinogenesis in rats. Cancer Res. 14, 407-410.

Haslam, E. (1981). In: The Biochemistry of plants, Vol 7, pp 527-533. Editor: E.V. Conn. Academic Press, New York, NY.

Hecht, S.S., Young, R., and Chen, C.B. (1980). Metabolism in the F344 rat of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, a tobacco-specific carcinogen. Cancer Res. 40, 4144-4150.

Hecht, S.S., Lin, D., and Chen, C.B. (1981). Comprehensive analysis of urinary metabolites of N-nitrosonornicotine. Carcinogenesis 2, 833-838.

Hecht, S.S., Lin, D., and Castonguay, A. (1983). Effects of α-deuterium substitution on the mutagenicity of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). Carcinogenesis 4, 305-310.

Hecht, S.S., and Hoffmann, D. (1988). Tobacco-specific nitrosamines, an important group of carcinogens in tobacco and tobacco smoke, Carcinogenesis 9, 875-884.

Hecht, S.S., and Hoffmann, D. (1989). The relevance of tobacco specific nitrosamines to human cancer. Cancer Surv. 8, 273-294.

Hecht S.S., Morse, M.A., Amin, S.G., Stoner, G.D., Jordan, K.G., Choi, C.I., and Chung, F.L. (1989). Rapid single dose model for lung tumor induction in A/J mice by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and the effect of diet. Carcinogenesis 10, 1901-1904.

Huang, M.T., Wood, A.W., Newmark, H.L., Sayer, J.M., Yagi, H., Jerina, D.M., and Conney, A.H. (1983). Inhibition of the mutagenicity by bay-region diol-epoxides of polycyclic aromatic hydrocarbons by phenolic plant flavonoids. Carcinogenesis 4, 1631-1637.

Imaida, K., Hirose, M., Shirai, T., and Ito, H. (1992). Inhibitory effect of green tea catechins in a rat multi-organ carcinogenesis model. Proc. Am. Assn. Cancer. Res. 33, 160.

Jorquera, R., Castonguay, A., and Schuller, H.M. (1992). Effects of pregnancy and ethanol treatment on the metabolism of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone by hamster liver and lung microsomes. Drug Metab. Dispos., 20, 510-517.

Kuehnau, J. (1976). The flavonoids. A class of semi-essential food components. Their role in human nutrition. World Rev. Nutr. Diet 24, 117-191.

Liu, L., and Castonguay, A. (1991). Inhibition of the metabolism and genotoxicity of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in rat hepatocytes by (+)-catechin. Carcinogenesis 12, 1203-1208.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951). Protein measurement with the folin phenol reagent. J. Biol. Chem. 192, 265-275.

Ludeke, B.I., Dominé, F., Ohgaki, H., and Kleihues, P. (1992). Modulation of N-nitrosomethylbenzylamine bioactivation by diallyl sulfide in vivo. Carcinogenesis 13, 2467-2470.

Mandal, S., Ahuya, A., Shivapurkar, N.M., Chen, S.J., Groopman, J.D, and Stoner, G.D. (1987). Inhibition of aflatoxin B_1 mutagenesis in *Salmonella typhimurium* and in F344 rats by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and N-nitrosodimethylamine. Cancer Res. 46, 498-502.

Maron, D.M., and Ames, B.N. (1983). Revised methods for the *Salmonella* mutagenicity test. Mutat. Res. 113, 173-215.

Maurya, A.K., and Singh, S.V. (1991). Differential induction of glutathione transferase isoenzymes of mice stomach by diallyl sulfide, a naturally occurring anticarcinogen. Cancer Lett. 57, 121-129.

Modly, C.E., Das, M., Don, P.S.C., Marcelo, C.L., Mukhtar, H., and Bickers, D. R. (1986). Capsaicin as an in vitro inhibitor of benzo[a]pyrene metabolism and its DNA binding in human and murine keratinocytes. Drug. Metab. Dispos. 14, 413-416.

Murphy, E., Palomino, A., Hecht, S.S., and Hoffmann, D. (1990). Dose-response study of DNA and hemoglobin adduct formation by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone in F433 rats. Cancer Res. 50, 5446-5452

Nagabhushan, M. and Bhide, S.V. (1988). Antimutagenicity of catechin against environmental mutagens. Mutagenesis 3, 293-296.

Newmark, H.L. (1984). A hypothesis for dietary components as blocking agents of chemical carcinogenesis: plant phenolics and pyrrole pigments. Nutr. Cancer 6, 58-70.

Okey, A.B. (1990). Enzyme induction in the cytochrome P450 system. Pharmac. Ther. 45, 241-298.

Peterson, L., Mathew, R., and Hecht, S.S. (1991). Quantitation of microsomal α-hydroxylation of the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone. Carcinogenesis 12, 2069-2072.

Rivensen, A., Hoffmann, D., Prokopczyk, B., Amin, S., and Hecht, S.S. (1988). Induction of lung and exocrine pancreas tumors in F344 rats by tobacco-specific and *Areca*-derived N-nitrosamines. Cancer Res. 48, 6912-6917.

Sayer J.M., Yagi, H., Wood, A.W., Conney, A.H., and Jerina, D.M. (1982). Extremely facile reaction between the ultimate carcinogen benzo[a]pyrene-7,8-diol 9,10-epoxide and reaction between the ultimate carcinogen benzo[a]pyrene-7,8-diol 9,10-epoxide and ellagic acid. J. Am. Chem. Soc. 104, 5562-5564.

Smith, T.J., Guo, Z., Thomas, P.E., Chung, F.L., Morse, M.A., Elkind, K., and Yang, C.S. (1990). Metabolism of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone in mouse lung microsomes and its inhibition by isothiocyanates. Cancer Res. 50, 6817-6822.

Sparnins, V.L., Barany, G., and Wattenberg, L.W. (1988). Effects of organosulfur compounds from garlic and onions on benzo[a]pyrene-induced neoplasia and glutathione S-transferase activity in the mouse. Carcinogenesis 9, 131-134.

Steele, V.E., Kelloff, G.J., Wilkinson, B.P., and Arnold, J.T. (1990). Inhibition of transformation in cultured rat tracheal epithelial cells by potential chemopreventive agents. Cancer Res. 50, 2068-2074.

Teel, R.W. (1991). Effects of capsaicin on rat liver S9-mediated metabolism and DNA-bindings of aflatoxin. Nutr. Cancer 15, 27-32.

Teel, R.W. (1992a). Modulation of microsomal activity by potential chemopreventive agents of plant origin. Phytotherapy Res. 6, 251-254.

Teel, R.W. (1992b). Effects of different inducers of cytochrome P450 on the mutagenesis of the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in *Salmonella typhimurium* TA1535. Anticancer Res. 12, 1287-1290.

Teel, R.W. (1993). Effect of phytochemicals on the mutagenicity of the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in *Salmonella typhimurium* strain TA1535. Phytotherapy Res. 7, 248-251.

Valenzuela, A., Guerra, R., and Videla, L.A. (1986). Antioxidant properties of the flavenoids silybin and cyanidanol-3: comparison with butylated hydroxyanisole and butylated hydroxytoluene. Planta Med. 6, 438-440.

Vance, R.E., and Teel, R.W. (1989). Effect of tannic acid on rat liver S9 mediated mutagenesis, metabolism and DNA binding of benzo[a]pyrene. Cancer Lett. 47, 37-44.

Wargovich, M.J. (1987). Diallyl sulfide, a flavor component of garlic (*Allium sativum*), inhibits dimethylhydrazine-induced colon cancer. Carcinogenesis 8, 487-489.

Wargovich, M.J., Woods, C., Eng, V.W.S., Stephans, L.C., and Gray, K. (1988). Chemoprevention of N-nitrosomethylbenzylamine-induced esophageal cancer in rats by the naturally occurring thioether, diallyl sulfide. Cancer Res. 48, 6872-6875.

Wattenberg, L.W. (1985). Chemoprevention of cancer. Cancer Res. 45, 1-8.

Wood, A.W., Huang, M.T., Chang, R.L., Newmark, H.L., Lehr, R.E., Yagi, H., Sayer, J. M., Jerina, D.M., and Conney, A.H. (1982). Inhibition of the mutagenicity of bay-region diol epoxides of polycyclic aromatic hydrocarbons by naturally occurring plant phenols: exceptional activity of ellagic acid. Proc. Natl. Acad. Sci. 79, 5513-5517.

CHAPTER V

EFFECTS OF CAPSAICIN ON LIVER MICROSOMAL METABOLISM OF THE TOBACCO-SPECIFIC NITROSAMINE NNK

Cecil H. Miller[1], Zuo Zhang[2], Solomon M. Hamilton[3], and Robert W. Teel[4]

[1] Graduate student who conducted the experiments and helped prepare the manuscript

[2] Graduate student in training who helped with some of the HPLC data collection

[3] Graduate student in training who helped with collecting HPLC samples and running the scintillation counter

[4] Student's research committee chairman who supervised the research and helped prepare the manuscript

Cancer Letters (1993); 75:45-52

EFFECTS OF CAPSAICIN ON LIVER MICROSOMAL METABOLISM OF THE TOBACCO-SPECIFIC NITROSAMINE NNK

C.H. Miller, Z. Zhang, S.M. Hamilton, and R.W. Teel[1]

[1]Department of Physiology and Pharmacology, School of Medicine, Loma Linda University, Loma Linda, CA 92350

Key Words: Capsaicin, cytochrome P450, metabolism, NNK

Correspondence: R.W. Teel, Ph.D.,
Department of Physiology and Pharmacology,
School of Medicine, Loma Linda University
Loma Linda, CA 92350 (U.S.A.)

SUMMARY

Chemically-induced mutagenesis and carcinogenesis is modulated by various plant products, some of which are present in the human diet. 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), a potent carcinogen in tobacco and tobacco smoke, is activated by microsomal enzymes. In this study, we investigated the effects of capsaicin on the *in vitro* metabolism of NNK. Capsaicin is the principal component of *Capsicum* fruits used widely by humans as a food additive. Liver microsomes from saline-injected, phenobarbital-induced and ß-naphthoflavone-induced hamsters were used. Microsomes from phenobarbital and ß-naphthoflavone-induced animals expressed decreased NNK-reduction and enhanced pyridine-N-oxidation, but did not significantly alter α-carbon hydroxylation of NNK. Capsaicin (0.5 mM) inhibited the formation of all metabolites of NNK by all microsomal fractions and inhibited α-hydroxylation by phenobarbital-induced microsomes more than by either of the other two treatments. Our results suggest that capsaicin, as a naturally occurring dietary constituent, possesses antimutagenic and

anticarcinogenic properties through the inhibition of xenobiotic metabolizing enzymes.

INTRODUCTION

Research results from various laboratories support the protective effect of certain naturally occurring phytochemicals toward chemically-induced mutagenesis and carcinogenesis [1,32]. Plant compounds with antimutagenic and/or anticarcinogenic actions may exert their effects on one or more of the many steps that take place during the initiation, promotion and progression phases of the cancer process. The chemopreventive properties of these compounds, many of which are present in foods humans normally consume, are attributed to one or more of these mechanisms: (a) modulation of the metabolic activation of a promutagen/procarcinogen to highly reactive species; (b) enhancement of detoxification; (c) blockage of the interaction of electrophilic metabolites with target macromolecules of the cell [24,28,31,33]; (d) suppression of the neoplastic process in cells [32].

4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), a nicotine-derived nitrosamine, is formed during the processing of tobacco [3]. It is present in tobacco smoke, to which both smokers and non-smokers are exposed [2,9]. NNK produces tumors in the

nasal mucosa, trachea, lungs, liver and pancreas of laboratory animals [15]. The targeting of lung tissue in these animals supports an etiological role of NNK in the causation of human lung cancer [14,15]. NNK requires metabolic activation by microsomal-dependent α-carbon hydroxylation reactions to produce mutagenic and carcinogenic properties [7,14]. The reactive electrophilic species methylate and pyridyloxobutylate purine bases of DNA [23]. A number of isozymes of cytochrome P450 are associated with the metabolic activation of NNK [7,8,11].

Nutrition affects the carcinogenic response to tobacco smoking [16] and thus reflects the relationship of diet to xenobiotic metabolism [34]. It is therefore important to identify naturally occurring compounds that can inhibit the mutagenic and carcinogenic action of NNK by a modulation of its metabolism [6,33]. Capsaicin (8-methyl-N-vanillyl-6-nonenamide) is a pungent, irritating ingredient of *Capsicum* fruits which are widely used in the world as a food additive [10]. Miller et al. [21] observed that capsaicin lengthened pentobarbital-induced sleep time in mice, suggesting an inhibition of drug metabolizing enzymes. In another study, a

dose-dependent inhibition of aryl hydrocarbon hydroxylase activity in epidermal microsomes correlated with an inhibition of the metabolism of benzo[a]pyrene in BALB/c mouse and human keratinocytes [22]. Capsaicin exhibited a dose-dependent inhibition of the mutagenesis of NNK in *Salmonella typhimurium* TA1535 [30].

Since the identification of naturally occurring chemicals with chemopreventive properties may eventually contribute to an effective and rational approach to the prevention of human cancers, we undertook *in vitro* studies to investigate the effects of capsaicin on the metabolism of NNK. The results are the subject of this report.

MATERIALS AND METHODS

Chemicals

[5-^{3}H]NNK (2.51 Ci/mmole) was purchased from Chemsyn Science Laboratory (Lenexa, KS). NNK metabolites used as reference standards were synthesized as reported [12,13]. NADP+, glucose-6-phosphate dehydrogenase, dimethylsulfoxide (DMSO) and capsaicin were purchased from Sigma Chemical Co., (St. Louis, MO).

Animals

Male Syrian golden hamsters weighing 150-160g (Charles River Laboratory, Wilmington, ME) were provided water and Purina rodent chow *ad libitum*. After three days the animals were randomly divided into three groups of four. One group was given daily i.p. injections of phenobarbital in saline (60 mg/kg) for five days. A second group was administered daily i.p. injections of ß-naphthoflavone in olive oil (80 mg/kg) for five days [25]. Control animals were given daily injections of saline. The animals were sacrificed 24 hrs after the last injection.

Preparation of microsomes

The livers were removed and homogenized in two volumes of 0.25M Tris-HCl buffer (pH 7.4). Microsomal fractions were obtained by differential centrifugation [20] and resuspended in Tris-HCl:sucrose:EDTA. Microsomal fractions from each treatment group were pooled, aliquoted and stored at -80°C. The protein content was determined by the method of Lowry et al. [19].

Assays of NNK metabolism

The procedure was a modification of that described by Smith et al. [27]. The incubation mixture contained 1 mM NADP+, 1 mM EDTA, 3 mM $MgCl_2$, 5 mM glucose-6-phosphate, 1.5 units glucose-6-phosphate dehydrogenase, 12.8 μCi [5-^{3}H]NNK, 5 mM sodium bisulfite, capsaicin (0.25, 0.5, 1.0 mM) in 50 μl DMSO in a final volume of one mL. These concentrations of capsaicin were non-toxic [30]. After a 10 min incubation in a shaking waterbath (37°C), the reaction was initiated by the addition of 0.5 mg hamster liver microsomal protein. Blanks were incubated with boiled microsomes and control incubations lacked capsaicin. All incubations contained

the same volume of DMSO. The reaction was terminated after 30 min by adding 200 µl of saturated barium sulfate and 200 µl 25% zinc sulfate. The precipitated samples were centrifuged at 14,000 x g for 20 min and the supernatant was filtered through 0.45 µm filters (MSI, Westboro, MA). One hundred µl aliquots of each filtered sample and 7 µl of NNK metabolite standards were injected onto a reversed-phase high pressure liquid chromatography (HPLC) system interfaced with a 10 µm µBondapak C18 column (3.9 x 300 mm, Millipore-Waters, Milford, MA). NNK and its metabolites were eluted with sodium acetate buffer (pH 6) and methanol [26]. Keto aldehyde was stabilized as keto aldehyde-bisulfite adducts in the presence of 1 mM sodium bisulfite in the elution buffer. The eluate was monitored at 254 nm and 1 ml fractions were collected. After the addition of scintillation cocktail (Scintiverse LC, Fisher Scientific, Tustin, CA), the radioactivity was expressed as pmoles/mg microsomal protein/min. Recovery of total radioactivity during HPLC analysis was > 70%.

Statistical analysis

Data were analyzed by the student's unpaired t-test using the MacIntosh Stat-View software program. Statistical significance was defined as $p < 0.05$ compared to control values.

RESULTS AND DISCUSSION

In this study, the effect of capsaicin on the metabolism of the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) was investigated using *in vitro* incubations of liver microsomes from saline-injected, PB-induced and ßNF-induced male hamsters. Isozymes of the CYP450 2B family induced by PB metabolize NNK. ßNF induces CYP450 1A isozymes which also activate NNK to mutagenic species [7].

The pathways in the metabolism of NNK are shown in Figure 1 [4,18]. Carbonyl reduction of NNK yields the N-nitroso alcohol NNAL (pathway a). N-oxidation of the pyridine ring of NNK and NNAL gives NNK-N-Oxide and NNAL-N-Oxide (pathway b). Methyl hydroxylation produces keto alcohol whereas α-methylene hydroxylation yields keto aldehyde (pathway c). The addition of 1 mM sodium bisulfite to the incubation mixture and HPLC eluant stabilizes the keto aldehyde as a keto aldehyde-bisulfite adduct [26]. The oxidation/reduction of keto alcohol and keto aldehyde yield keto acid, hydroxy acid and diol as metabolites.

Figure1: Metabolic pathways of NNK: carbonal reduction (pathway a), pyridine N-oxidation (pathway b), and α-carbon hydroxylation (pathway c). Metabolites: NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; NNK-N-oxide, 4-(methylnitrosamino)-1-(3-pyridyl-N-oxide)-1-butanone; NNAL-N-oxide, 4-(methylnitrosamino)-1-(3-pyridyl-N-oxide)-1-butanol; keto alchol, 4-oxo-4-(3-pyridyl)-1-butanol; hydroxy acid, 4-hydroxy-4-(3-pyridyl) butyric acid; keto aldehyde, 4-oxo-4-(3-pyridyl) butanal; keto acid, 4-oxo-4-(3-pyridyl) butyric acid; diol, 4-hydroxy-1-(3-pyridyl)-1-butanol.
The structures in brackets are hypothetical intermediates.
(Adapted from Jorquera et al., 1992; Hecht et al., 1980).

NNK

NNAL

1

NNK - N - Oxide

2

NNAL - N - Oxide

3

4

5

6

7

+ HCHO → CO_2

8

Keto aldehyde

+ [$CH_3N=NOH$]

9 → CH_3OH

10

+ 9 → CH_3OH

11

+ HCHO → CO_2

12

Keto alcohol

13

Keto acid

14

Hydroxy acid

15

Diol

The levels of metabolites formed by liver microsomes from saline-injected (SI), phenobarbital (PB)-induced and ß-naphthoflavone (ßNF)-induced hamsters are shown in Table 1 and Figure 2. NNK reduction (pathway a, Figure 1) was significantly less by microsomes from PB-induced and ßNF-induced animals compared to microsomes from saline-injected (SI) controls. N-oxidation of the pyridine ring of NNK and NNAL (pathway b, Figure 1) was enhanced by microsomes from PB-induced and ßNF induced animals. α-hydroxylation (pathway c, Figure 1) was not significantly affected by either PB or ßNF induction. Methyl hydroxylation leading to keto alcohol was much more pronounced than α-methylene hydroxylation and the formation of keto-aldehyde by all the microsomes (Table 1). Microsomes from both PB and ßNF-induction showed enhanced methyl hydroxylation compared to microsomes from SI animals, although only ßNF-induction showed a statistically significant difference. Methylating agents are reported to alkylate DNA more efficiently than pyridyloxobutylating agents [14].

Capsaicin was a potent inhibitor of the metabolism of NNK by microsomes from SI hamsters at all concentrations tested

Table 1. A comparison of the metabolism of NNK by liver microsomes from saline-injected, phenobarbitol-induced and ß-naphthoflavone-induced hamsters.

METABOLITE[a]	Microsome Sample[b]		
pmol/mg/min[c]	Saline-injected	PB-induced	ß-NF-induced
Hydroxy acid (c)	N.D.	1.51 ± 0.1*	0.21 ± 0.1
Keto acid (c)	12.4 ± 0.52	5.44 ± 0.09*	3.76 ± 0.54*
Diol (c)	10.35 ± 0.54	11.4 ± 1.5	9.12 ± 0.69
Keto alcohol (c)	41.52 ± 0.6	48.85 ± 6.0	52.5 ± 0.73*
Keto aldehyde[d] (c)	0.56 ± 0.05	0.39 ± 0.13	0.67 ± 0.14
NNAL-N-oxide (b)	7.43 ± 1.15	12.03 ± 0.94*	12.27 ± 0.3*
NNK-N-oxide (b)	5.16 ± 0.33	28.0 ± 0.4*	18.1 ± 0.5*
NNAL (a)	82.13 ± 3.0	68.7 ± 0.53*	47.2 ± 0.23*
Total (b)	12.58 ± 0.84	40.03 ± 0.98*	30.38 ± 0.28*
Total (c)	64.83 ± 0.47	64.24 ± 3.82	70.39 ± 3.72
% unmetabolized			
NNK + NNAL	50.42 ± 1.4	41.65 ± 0.55*	44.0 ± 0.6*

[a]Refer to Figure 1 for names of metabolites and metabolic pathways (given in parentheses).

[b]In vitro incubations of 0.5 mg liver microsome protein with [^{3}H]NNK were performed as described in Materials and Methods.

[c]Values are the mean ± S.E.M. from three determinations. N.D. = not detected.

[d]Keto aldehyde was detected as the keto aldehyde-bisulfite adduct.

*Significantly different ($p \leq 0.05$) from saline-injected (noninduced) controls.

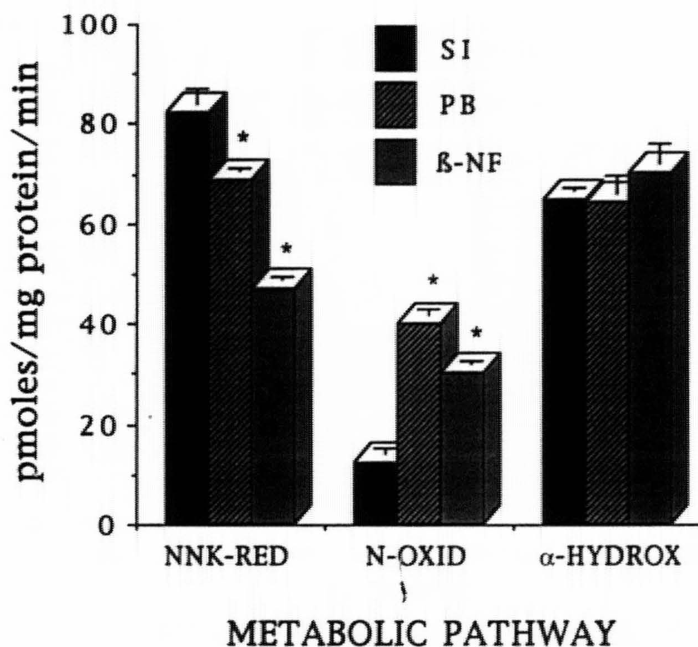


Figure 2: Metabolism of NNK by liver microsomes from saline-injected (SI), phenobarbitol-induced (PB) and ß-naphthoflavone-induced (ß-NF) hamsters. Total NNK-reduction, total N-oxidation and total α-carbon hydroxylation from Table 1 are shown. Asterisks indicate a statistically significant difference from saline-injected controls, $p < 0.05$.

(Figure 3, Table 2). The inhibition affected NNK-reduction, N-oxidation and α-hydroxylation. This effect was also observed with microsomes from PB and ßNF-induced animals (Figure 4, Table 3). This suggests that capsaicin inhibited isozymes of the CYP450 1A and 2B families. Capsaicin inhibited α-hydroxylation by PB-induced microsomes more than by microsomes from ßNF-induced or SI hamsters.

Capsaicin is reported to have a competitive inhibitory-like effect on ethylmorphine-N-demethylase activity in liver microsomes [17]. Its effect on epidermal microsomal aryl hydrocarbon hydroxylase is described as noncompetitive inhibition [22]. Capsaicin produces a type I spectral change in rat liver microsomes and is almost as potent as SKF-525A in inhibiting ethylmorphine demethylation [21]. We have previously reported the inhibition of the *in vitro* metabolism and DNA binding of aflatoxin B_1 and the inhibition of the mutagenicity of NNK in *Salmonella typhimurium* TA1535 by capsaicin [29,30].

Chemoprevention of mutagenesis and carcinogenesis is reported to occur through one or more of the following mechanisms:

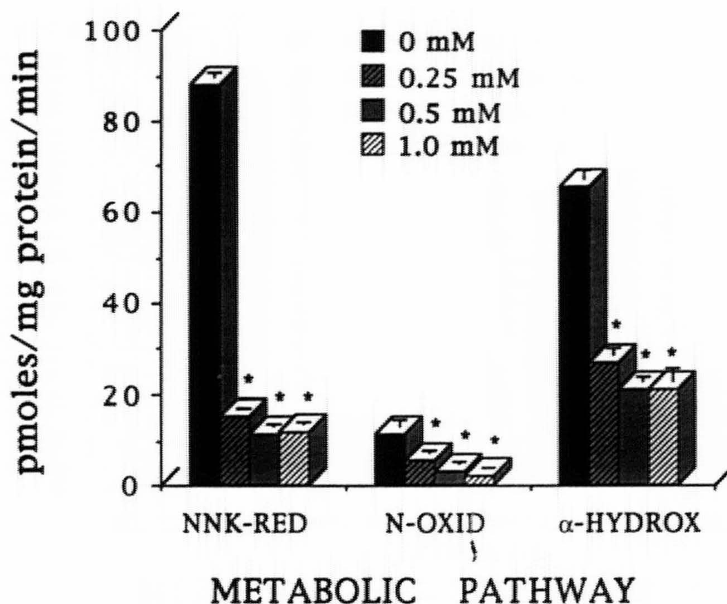


Figure 3: Effect of concentration of capsaicin on the metabolism of NNK by liver microsomes from saline-injected hamsters. Total NNK reduction, total pyridine N-oxidation, and total α-carbon hydroxylation fron Table 2 are shown. Asterisks indicate a statistically significant difference from control (0 mM), $p < 0.05$.

Table 2. Effect of concentration of capsaicin on the metabolism of NNK by liver microsomes from saline-injected hamsters[b].

METABOLITE[a]	Concentration capsaicin, mM			
pmol/mg/min[c]	0	0.25	0.5	1.0
Hydroxy acid (c)	N.D.	N.D.	N.D.	N.D.
Keto acid (c)	9.1 ± 1.3	5.23 ± 0.97	1.46 ± 0.44*	0.4 ± 0.03*
Diol (c)	8.9 ± 0.6	3.66 ± 0.08*	1.68 ± 0.12*	1.9 ± 0.2*
Keto alcohol (c)	46.91 ± 0.52	17.6 ± 0.6*	17.7 ± 1.5*	18.7 ± 2.9*
Keto aldehyde[d] (c)	0.45 ± 0.03	0.17 ± 0.05*	N.D.*	N.D.*
NNAL-N-oxide (b)	6.11 ± 0.5	3.66 ± 0.08*	2.54 ± 0.28*	1.93 ± 0.2*
NNK-N-oxide (b)	5.34 ± 0.54	1.65 ± 0.37*	0.46 ± 0.44*	N.D.*
NNAL (a)	87.7 ± 1.22	15.0 ± 0.21*	11.3 ± 0.36*	11.8 ± 0.24*
Total (b)	11.41 ± 1.03	5.31 ± 0.4*	2.96 ± 0.46*	1.93 ± 0.2*
Total (c)	65.4 ± 1.8	26.7 ± 1.64*	20.87 ± 1.08*	21.1 ± 2.82*
% unmetabolized NNK + NNAL	50.13 ± 0.53	75.83 ± 0.7*	84.9 ± 0.59*	83.96 ± 0.47*

[a]Refer to Figure 1 for names of metabolites and metabolic pathways (given in parentheses).

[b]In vitro incubations of 0.5 mg liver microsome protein with $[^3H]$NNK were performed as described in Materials and Methods.

[c]Values are the mean ± S.E.M. from three determinations. N.D. = not detected.

[d]Keto aldehyde was detected as the keto aldehyde-bisulfite adduct.

*Significantly different ($p \leq 0.05$) from control value.

Figure 4. Effect of capsaicin (0.5 mM) on the metabolism of NNK by hamster liver microsomes. Total NNK-reduction, total N-oxidation and total α-hydroxylation from Table 3 is shown. A = saline-injected control; B = phenobarbital-induced; C = ß-naphthoflavone-induced. Asterisks indicate statistically significant difference from control, $p < 0.05$.

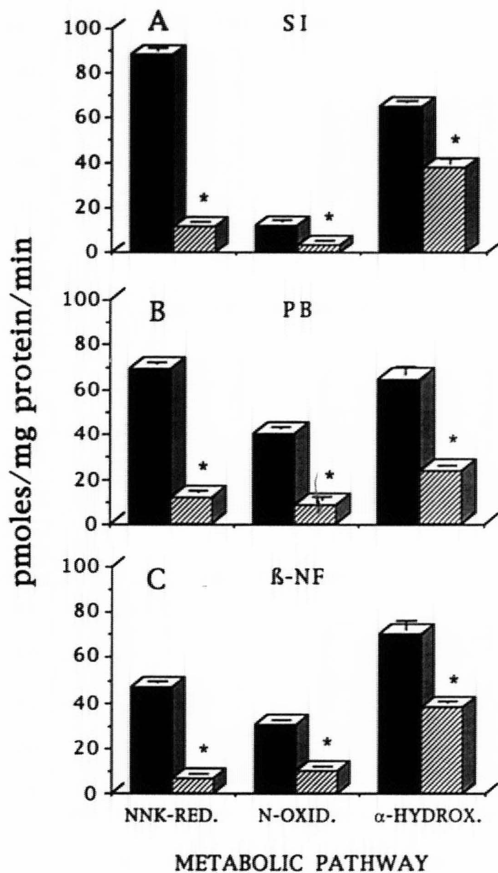

A
S I
B
P B
C
ß-NF
pmoles/mg protein/min
100
80
60
40
20
0
NNK-RED.
N-OXID.
α-HYDROX.
METABOLIC PATHWAY

Table 3. Effect of capsaicin on the metabolism of NNK by hamster liver microsomes.

METABOLITE[a] pmol/mg/min[c]	TREATMENT[b]					
	Saline-injected		PB-induced		ß-NF-induced	
	0 mM	0.5 mM	0 mM	0.5 mM	0 mM	0.5 mM
Hydroxy acid (c)	N.D.	N.D.	1.51 ± 0.1	0.36 ± 0.17*	0.21 ± 0.01	0.57 ± 0.06*
Keto acid (c)	9.1 ± 1.3	1.5 ± 0.44*	5.44 ± 0.1	1.64 ± 0.5*	3.76 ± 0.54	1.26 ± 0.5*
Diol (c)	8.9 ± 0.6	1.7 ± 0.12*	11.4 ± 1.5	3.9 ± 0.48*	9.12 ± 0.69	2.52 ± 0.19*
Keto alcohol (c)	46.9 ± 0.52	17.7 ± 1.5*	48.85 ± 6.0	16.83 ± 0.57*	52.47 ± 0.73	34.02 ± 0.9*
Keto aldehyde[d] (c)	0.45 ± 0.03	N.D.*	0.39 ± 0.13	N.D.*	0.67 ± 0.14	N.D.*
NNAL-N-oxide (b)	6.11 ± 0.51	2.54 ± 0.28*	12.03 ± 0.94	5.35 ± 0.99*	12.27 ± 0.28	2.87 ± 0.12*
NNK-N-oxide (b)	5.34 ± 0.54	0.46 ± 0.44*	27.99 ± 0.4	3.04 ± 0.35*	18.1 ± 0.51	7.0 ± 0.37*
NNAL (a)	87.7 ± 1.22	11.3 ± 0.36*	68.96 ± 0.53	12.12 ± 0.21*	47.2 ± 0.23	6.5 ± 0.08*
Total (b)	11.45 ± 0.84	3.0 ± 0.11*	40.02 ± 0.98	8.38 ± 1.32*	30.38 ± 0.28	9.86 ± 0.31*
Total (c)	64.83 ± 0.47	37.54 ± 2.3*	64.24 ± 3.82	23.56 ± 0.16*	70.39 ± 3.72	38.39 ± 0.39*
% unmetabolized						
NNK + NNAL	50.13 ± 0.52	84.9 ± 0.6*	41.65 ± 0.55	73.5 ± 0.55*	44.0 ± 0.6	66.6 ± 0.75*

[a]Refer to Figure 2 for names of metabolites and metabolic pathways (given in parentheses).

[b]In vitro incubations of 0.5 mg liver microsome protein from saline-injected, ß-NF-induced and PB-induced hamsters with [^{3}H]NNK were performed in the absence (0 mM) or presence of 0.5 mM capsaicin as described in Materials and Methods.

[c]Values are the mean ± S.E. from two determinations. N.D. = not detected.

[d]Keto aldehyde was detected as the keto aldehyde-bisulfite adduct.

*Significantly different ($p \leq 0.05$) from control value

inhibition of the enzymatic activation of promutagens and procarcinogens to reactive intermediates [24]; enhancement of detoxification pathways [32]; blockage of the interaction of reactive intermediates with DNA [24] and/or suppression of the neoplastic state [32]. Results described in this study support the chemopreventive action of capsaicin and suggest that capsaicin may play a role in dietary protection against carcinogens in the environment including the tobacco-specific nitrosamine NNK. Our results also suggest that a mechanism by which capsaicin exerts its chemopreventive effect is through an inhibition of the activity of cytochrome P450.

ACKNOWLEDGEMENTS

We wish to thank Velma Clem and Jewelyn Gallego for their assistance in preparation of this manuscript.

REFERENCES

1. Bertram, J.S., Kolonel, N.N. and Meyskens, F.L. (1987) Rationale and strategies for chemoprevention of cancers in humans. Cancer Res., 47, 3012-3031.

2. Brunnemann, K.D., Cox, J.E. and Hoffmann, D. (1992) Analysis of tobacco-specific N-nitrosamines in indoor air. Carcinogenesis, 13, 2415-2418.

3. Burton, H.R., Childs, G.H., Anderson, R.A. and Fleming, P.D. (1989) Changes in chemical composition of Burley tobacco during senescence and curing. 3. Tobacco-specific nitrosamines. J. Agric. Food Chem., 37, 426-430.

4. Castonguay, A., Tjalve, H., Trushin, N. and Hecht, S.S. (1984) Perinatal metabolism of the tobacco-specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone in C57BL mice. J. Natl. Cancer Inst., 72, 1117-1124.

5. Castonguay, A., Pepin, P. and Briere, N. (1991) Modulation of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone demethylation and denitrosation by rat liver microsome. Cancer Lett., 59, 67-74.

6. Chung, F-L. (1992) Chemoprevention of lung carcinogenesis by aromatic isothiocyanates. In: Cancer Chemoprevention. Ed. by L. Wattenberg, M. Lipkin, C.W. Boone and G. J. Kelloff, pp 227-245. CRC Press, Boca Raton, FL.

7. Crespi, C.L., Penman, B.W., Gelboin, H.V. and Gonzalez, F.J. (1991) A tobacco smoke derived nitrosamine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, is activated by multiple human cytochrome P450s including polymorphic human cytochrome P450 2D6. Carcinogenesis, 12, 1197-1201.

8. Devereux, T.R., Anderson, M.W. and Belinsky, S.A. (1988) Factors regulating activation and DNA alkylation by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and nitrosodimethylamine in rat lung and isolated lung cells and the relationship to carcinogenicity. Cancer Res. 48, 4215-4221.

9. Fischer, S., Spiegelhalder, B., Eisenbarth, J., Preussmann, R. (1990) Investigations on the origin of tobacco-specific nitrosamines in mainstream smoke of cigarettes. Carcinogenesis, 11, 723-730.

10. Govindarajan, V.S. and Sathyanarayana, M.N. (1991) Capsicum-production, technology, chemistry and quality. Part V. Impact on physiology, pharmacology, nutrition and metabolism; structure, pungency, pain and desensitization sequences. Critical Reviews in Food Sci. and Nutr., 29, 435-474.

11. Guo, Z., Smith, T.J., Ishizaki, H. and Yang, C.S. (1991) Metabolism of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) by cytochrome P450 2B1 in a reconstituted system. Carcinogenesis, 12, 2277-2282.

12. Hecht, S.S., Young, R. and Chen, C.B. (1980) Metabolism in the F344 rat of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, a tobacco-specific carcinogen. Cancer Res., 40, 4144-4150.

13. Hecht, S.S., Lin, D. and Chen, C.B. (1981) Comprehensive analysis of urinary metabolites of N-nitrosonornicotine. Carcinogenesis, 2, 833-838.

14. Hecht, S.S. and Hoffmann, D. (1988) Tobacco-specific nitrosamines, an important group of carcinogens in tobacco and tobacco smoke. Carcinogenesis, 9, 875-884.

15. Hecht, S.S. and Hoffmann, D. (1989) The relevance of tobacco-specific nitrosamines to human cancer. Cancer Surv., 8, 273-294.

16. Hirayama, T. (1984) Lung Cancer in Japan: Effects of nutrition and passive smoking. In: Lung Cancer: causes and prevention. Ed. by M. Mizell and P. Corres. pp. 175-195. Verlag Chemie International, Inc., Deerfield Beach, Fl.

17. Jhamandes, K., Yaksh, T.L., Haty, G., Szolcsanyi, J. and Go, V.L.W. (1984) Action of intrathecal capsaicin and its structural analogues on the content and release of spinal substance P: selectivity of action and relationship to analgesia. Brain Res., 306, 215-225.

18. Jorquera, R. and Castonguay, A. (1992) Effects of pregnancy and ethanol treatment on the metabolism of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone by hamster liver and lung microsomes. Drug Metab. Dispos., 20, 510-517.

19. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) Protein measurement with the Folin phenol reagent. J. Biol. Chem., 192, 265-275.

20. Maron, D.M. and Ames, B.N. (1983) Revised methods for the *Salmonella* mutagenicity test. Mutat. Res., 113, 173-215.

21. Miller, M.S., Brendel, K., Burks, T.F. and Sipes, I.G. (1983) Interaction of capsaicinoids with drug-metabolizing systems. Biochem. Pharmacol., 32, 547-551.

22. Modley, C.E., Das, M., Don, P.S.C., Marcelo, C.L., Mukhtar, H. and Bickers, D.R. (1986) Capsaicin as an in vitro inhibitor of benzo[a]pyrene metabolism and its DNA binding in human and murine keratinocytes. Drug Metab. Dispos. 14, 413-416.

23. Murphy, S.E., Palomino, A., Hecht, S.S. and Hoffmann, D. (1990) Dose-response study of DNA and hemoglobin adduct formation by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone in F344 rats. Cancer Res., 50, 5446-5452.

24. Newmark, H.L. (1984) A hypothesis for dietary components as blocking agents of chemical carcinogenesis: plant phenolics and pyrrole pigments. Nutr. Cancer, 6, 58-70.

25. Paolini, M., Sapigni, E., Hrelia, P., Scotti, M., Morotti, M. and Cantelli-Forti, G. (1991) Wide spectrum detection of precarcinogens in short-term bioassays by simultaneous superinduction of multiple forms of cytochrome P450 isoenzymes. Carcinogenesis, 12, 759-766.

26. Peterson, L.A., Mathew, R. and Hecht, S.S. (1991) Quantation of microsomal α-hydroxylation of the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone. Carcinogenesis, 12, 2069-2072.

27. Smith, T.J., Guo, Z., Thomas, P.E., Chung, F.L., Morse, M.A., Elkind, K. and Yang, C.S. (1990) Metabolism of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone in mouse lung microsomes and its inhibition by isothiocyanates. Cancer Res., 50, 6817-6822.

28. Taladay, P., Delong, M.J. and Prochaska, H.J.(1988) Identification of a common chemical signal regulating the induction of enzymes that protect against chemical carcinogenesis. Proc. Natl. Acad. Sci., 85, 8261-8265.

29. Teel, R.W. (1991) Effects of capsaicin on rat liver S9-mediated metabolism and DNA binding of aflatoxin. Nutr. Cancer, 15, 27-32.

30. Teel, R.W. (1993) Effect of phytochemicals on the mutagenicity of the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in *Salmonella typhimurium* Strain TA 1535. Phytother. Res., 7, 248-251.

31. Wattenberg, L.W. and Lam, L.K.T. (1981) Inhibition of chemical carcinogenesis by phenols, coumarins, aromatic isocyanates, flavones and indoles. In: Inhibition of Tumor Induction and Development, Ed. by M.S. Zedeck and M. Lipkin, pp. 1-22. Plenum Press, New York, N.Y.

32. Wattenberg, L.W. (1985) Chemoprevention of cancer. Cancer Res., 45, 1-8.

33. Wattenberg, L.W. (1992) Chemoprevention of cancer by naturally occurring and synthetic compounds. In: Cancer Chemoprevention. Ed. by L. Wattenberg, M. Lipkin, C. W. Boone and G. J. Kelloff, pp 19-39. CRC Press, Boca Raton, FL.

34. Yang, C.S., Brady, J.F. and Hong, J-Y (1992) Dietary effects on cytochrome P450, xenobiotic metabolism and toxicity. FASEB J., 6, 737-744.

CHAPTER VI

DISCUSSION

Following the Introduction chapter, the results from experiments that were performed to determine the efficacy of 10 phytochemicals as chemopreventive agents for NNK-induced mutagenesis and carcinogenesis are described. These kinds of studies are necessary because cancer is currently the second leading cause of death in the United States. It is projected that by the year 2000 it will become the leading cause. Currently about 7 million Americans have cancer. Not only does this disease bring suffering and tragedy to the families with cancer, it carries an exorbitant financial burden as well. Current estimates indicate that cancer related costs in the US are over 80 billion dollars annually [Weinstein, 1991].

Tobacco related cancers are theoretically the most preventable cancers. The simplest prevention is cessation of tobacco use. Statistics, however, indicate that 56 million Americans still smoke in spite of the known health risks. Global estimates indicate that there are 2.5 million tobacco related deaths per year and by the year 2050 projections indicate that this will increase to 10 million [Ries et al., 1991; Weinstein, 1991]. Smoking cessation programs are not sufficient to prevent all tobacco related cancers. The identification of chemicals with chemopreventive properties to reduce cancer

incidence for those individuals that are unable to overcome their nicotine addiction is desirable.

The experimental approach described in these 2-4 chapters include the use of the Salmonella/mammalian microsome/mutagenesis assay [Maron and Ames, 1983] to determine the effectiveness of each phytochemical in inhibiting NNK-induced mutagenesis. In addition, the effects of each phytochemical on the microsomal-mediated metabolism of NNK was determined by high-performance liquid chromatography and liquid-scintillation spectroscopy. Chapter 2 describes preliminary experiments to optimize the conditions for NNK-induced mutagenesis in *Salmonella typhimurium* TA1535. The results of these experiments indicated that the master plates and agar plates should be one week old or less, that the concentration of NNK be 80 mM in saline, that 1.0 mg of microsomal protein should be used and that the amount of DMSO used should be minimized.

Chapter 3 describes results of studies of the effect of five phenolic phytochemicals on the mutagenesis and metabolism of NNK using microsomes from untreated (control), phenobarbitol (PB), and ß-naphthoflavone (ß-NF) treated hamsters. Western blot analysis of the microsomal protein using polyclonal antibodies specific for P450 1A2 and P450 2B1/2B2 confirmed that PB-induced microsomes expressed elevated amounts of P450 2B1/2B2 and that ß-NF-induced microsomes expressed elevated P450 1A2. The induced microsomes enhanced both the mutagenicity and metabolism of NNK. The

inhibition of the mutagenicity of NNK by the phenolic compounds correlated to some extent with their effect on the metabolism of NNK. Inhibition of mutagenesis was dependent upon the enzyme induction treatment. Ellagic acid was the most efficient inhibitor with untreated (control) microsomes while propyl gallate was the most efficient with PB and ß-NF-induced microsomes.

Chapter 4 describes studies of the effects of five non-phenolic phytochemicals on the microsome-mediated mutagenicity and metabolism of NNK. d-Limonene and silymarin had no effect on NNK-induced mutagenesis in *Salmonella typhimurium* TA1535. Diallyl sulfide was weakly antimutagenic at the highest concentration tested. Both capsaicin and tannic acid exhibited a dose-dependent inhibition of mutagenesis. The NNK metabolism studies showed that d-limonene and silymarin had the least inhibitory effect on total α-carbon hydroxylation (19 and 28% respectively). Diallyl sulfide and capsaicin inhibited these pathways by 70 and 74% respectively. Tannic acid, the most potent phytochemical tested, inhibited total α-carbon hydroxylation by 99%.

Chapter 5 details the studies of the effects of capsaicin, the principal component of hot chili peppers, on the metabolism of NNK by microsomes from untreated, PB and ß-NF-treated hamsters. This phytochemical inhibited NNK-reduction, N-oxidation and α-hydroxylation by all the microsome fractions tested. Capsaicin inhibited α-hydroxylation by PB-induced microsomes more than by either of the other two treatments.

It is of interest to note that several of the phytochemicals that exhibited antimutagenicity towards NNK are present in various foods humans normally eat. These include capsaicin, catechin, ellagic acid, d-limonene, tannic acid and diallyl sulfide. This supports a relationship of diet to the metabolism of xenobiotics [Yang et al., 1992]. Three of these phytochemicals exhibited strong antimutagenicity toward NNK that corresponded with an inhibition of α-carbon hydroxylation of NNK. The mutagenic and carcinogenic properties of NNK are dependent upon α-hydroxylation [Crespi et al., 1991; Hecht and Hoffmann, 1988]. Several isozymes of cytochrome P450 have been associated with the metabolic activation of NNK [Crespi et al., 1991; Devereux et al., 1988; Guo et al., 1991]. Our results suggest that certain phytochemicals exhibit selective affinities for specific P450 isozymes that activate NNK. Although the studies described are limited to *in vitro* experiments, they support the importance of the identification and characterization of naturally occurring compounds with chemopreventive properties that could eventually contribute to an effective and rational approach to the prevention of human cancers.

REFERENCES

Crespi CL, Penman BW, Gelbroin HV, Gonzalez F-J (1991): A tobacco smoke-derived nitrosamine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, is activated by multiple human cytochrome P450s including polymorphic human cytochrome P450 2D6. Carcinogenesis 12:1197-1201.

Devereux TR, Anderson MW and Belinsky SA (1988): Factors regulating activation and DNA alkylation by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and nitrosodimethylamine in rat lung and isolated lung cells and the relationship to carcinogenicity. Cancer Res 48:4215-4221.

Guo Z, Smith TJ, Ishizaki H, Yang CS (1991): Metabolism of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) by cytochrome P450 2B1 in a reconstituted system. Carcinogenesis 12:2277-2282.

Hecht SS, Hoffmann D (1988): Tobacco-specific nitrosamines, an important group of carcinogens in tobacco and tobacco smoke. Carcinogenesis 9:875-884.

Maron DM, Ames BN (1993): Revised methods for the Salmonella mutagenicity test. Mutation Res 113:173-215.

Ries LAG, Hankey BF, Miller BA, Hartman AM, Edwards BK (1991): Cancer statistics review 1973-88. National Cancer Institute NIH Pub. No. 91-2789.

Weinstein IB (1991): Cancer prevention: recent progress and future opportunities. Cancer Res 51:5080s-5085s.

Yang CS, Brady JF, Hong J-Y (1992): Dietary effects on cytochrome P450, xenobiotic metabolism and toxicity. FASEB J 6:737-744.

ABSTRACT

INHIBITION OF NNK MUTAGENESIS AND METABOLISM BY CHEMOPREVENTIVE PHYTOCHEMICALS

by

Cecil Henry Miller

Approximately one third of all cancer deaths in the United States can be linked to the use of tobacco products. Cessation of smoking is not always an effective prevention method, especially since nicotine is so addictive. For those individuals who continue to use tobacco products, chemoprevention may be an alternative to reduce their cancer risk. We have investigated the chemopreventive efficacy of five phenolic (catechin, ellagic acid, esculetin, esculin, and propyl gallate) and five non-phenolic (capsaicin, silymarin, diallyl sulfide, tannic acid, and d-limonene) phytochemicals on tobacco-specific nitrosamine (NNK)-induced mutagenesis and NNK metabolism. In the mutagenesis studies, *Salmonella typhimurium* strain TA1535 was used in the Ames/mammalian microsome/mutagenesis assay. In the initial studies, we determined that the optimal conditions for NNK-induced mutagenesis included the use of master plates and agar plates that were less than one week old, saline as a solvent for NNK, 1 mg hamster liver microsomal protein, and minimal DMSO. Experimental results showed that NNK-induced mutagenesis and metabolism of NNK was enhanced by microsomes from both

phenobarbitol (PB) and ß-naphthaflavone (ß-NF) treated hamsters and that the effect of the phenolic compounds on NNK-induced mutagenesis depended upon treatment. Ellagic acid was the most efficient inhibitor of NNK-induced mutagenesis by microsomes from untreated animals (controls) while propyl gallate was the most effective inhibitor of mutagenesis by microsomes from PB and ß-NF treated animals. The effect of the phenolic compounds on the microsome-mediated metabolism of NNK correlated with the effect on mutagenesis to some extent. Capsaicin and tannic acid were the most potent inhibitors of NNK-induced mutagenesis of the non-phenolic phytochemicals. Diallyl sulfide was weakly antimutagenic and d-limonene and silymarin had no effect. All five non-phenolic compounds inhibited the α-carbon hydroxylation pathway of NNK metabolism. Studies of the effect of capsaicin on NNK metabolism by microsomes from untreated, PB-treated and ß-NF-treated animals showed that capsaicin inhibited the formation of all metabolites of NNK by all microsomal fractions.