INVESTIGATION OF THE BIOTRANSFORMATION OF
4-(METHYLNITROSAMINO)-1-(3-PYRIDYL)-1-BUTANONE
BY PROSTAGLANDIN H SYNTHASE AND CYTOCHROME P450 2F

by

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Abstract

The tobacco-specific nitrosamine 4-(methylnitrosamo)-1-(3-pyridyl)-1-butanone (NNK) is believed to play a role in human lung cancer induced by tobacco smoking. NNK biotransformation may involve the enzymes prostaglandin H synthase (PHS)-1, PHS-2 and cytochrome 450 (CYP) 2F. PHS activity is thought to be important in extrahepatic tissues, where CYP activity is low. The CYP2F subfamily contains a single functional enzyme in humans (CYP2F1) and goats (CYP2F3); these enzymes are preferentially expressed in the lung, with little or no expression in other organs. The role of these enzymes in the pulmonary biotransformation of NNK was investigated.

4.2 µM [5-3H]NNK was incubated with human lung microsomes under NADPH-dependent and arachidonic acid-dependent conditions. Metabolites reflective of NNK α-carbon hydroxylation, N-oxidation and carbonyl reduction were detected in the presence of NADPH, and metabolite levels for all three biotransformation pathways were lower in the presence of arachidonic acid compared with NADPH (p<0.05, N=4). Incubation of microsomes with the PHS-1 selective inhibitor SC-560 and the PHS-2 selective inhibitor NS-398 did not change NNK biotransformation either in the presence of NADPH or in the presence of arachidonic acid (p>0.05, N=4). Incubation of [5-3H]NNK with ovine PHS-1 or PHS-2 did not result in formation of α-carbon hydroxylation or N-oxidation metabolites; 4-(methylnitrosamo)-1-(3-pyridyl)-1-butanol (NNAL) was measurable only in the presence of PHS-2.
Incubation of goat recombinant CYP2F3 with [5-\(^3\)H]NNK resulted in formation of keto acid, keto alcohol and NNK-\(N\)-oxide (65.0%, 17.5% and 30.0% (\(\mu\)mol enzyme\(^{-1}\) minute\(^{-1}\)), respectively). Metabolite formation was inhibited by 3-methylindole (3-MI), a mechanism-based inactivator of CYP2F3. Based on an \(N\) value of 3, incubation of human lung microsomes with 3-MI inhibited \(N\)-oxidation (\(p<0.05\)) but did not alter NNK bioactivation or carbonyl reduction (\(p>0.05\)). However, when metabolite formation was examined in lung microsomes from different individuals, decreases in NNK biotransformation (ranging from 19.6 to 68.5%) were observed and were more pronounced in some patients than others, suggesting inter-individual variability in CYP2F1 activity.

These studies demonstrate the ability of CYP2F to biotransform NNK and suggest inter-individual variability in the importance of CYP2F1 for this activity in human lung. They also strongly argue against the involvement of PHS enzymes.

**Keywords:** 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; biotransformation; pulmonary carcinogenesis; prostaglandin H synthases; cytochrome P450; human lung.
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<tr>
<td>[5-(^{3}\text{H})]</td>
<td>Tritium Covalently Bonded to Carbon-5 of a Chemical Compound</td>
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<tr>
<td>3-MI</td>
<td>3-Methylindole</td>
</tr>
<tr>
<td>3-MEI</td>
<td>3-Methyleneindolenine</td>
</tr>
<tr>
<td>3-MOI</td>
<td>3-Methyloxindole</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>B[a]P</td>
<td>Benzo[a]pyrene</td>
</tr>
<tr>
<td>BER</td>
<td>Base Excision Repair</td>
</tr>
<tr>
<td>BP-7,8-diol</td>
<td>(±)-7,8-dihydroxy-7,8-dihydrobenzo[a]pyrene</td>
</tr>
<tr>
<td>CYP450</td>
<td>Cytochrome P450</td>
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<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic Acid</td>
</tr>
<tr>
<td>Fe</td>
<td>Iron</td>
</tr>
<tr>
<td>H(_2)O(_2)</td>
<td>Hydrogen Peroxide</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric Acid</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
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<tr>
<td>I3C</td>
<td>Indole-3-carbinole</td>
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<tr>
<td>KCl</td>
<td>Potassium Chloride</td>
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<td>LOX</td>
<td>Lipoxygenase</td>
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<td>MgCl(_2)</td>
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<td>Nitrogen</td>
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<tr>
<td>NADP</td>
<td>Nicotinamide Adenine Dinucleotide Phosphate</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide Adenine Dinucleotide Phosphate, reduced form</td>
</tr>
<tr>
<td>N/D</td>
<td>Not Detectable</td>
</tr>
<tr>
<td>NER</td>
<td>Nucleotide Excision Repair</td>
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<td>NNAL</td>
<td>4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanol</td>
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<td>NNK</td>
<td>4-(Methylnitrosamino)-1-(3-pyridyl)-1-butaneone</td>
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<tr>
<td>NS-398</td>
<td>N-[2-(Cyclohexyloxy)-4-nitrophenoxy]methanesulfonamide</td>
</tr>
<tr>
<td>PG</td>
<td>Prostaglandin</td>
</tr>
<tr>
<td>PGG(_2)</td>
<td>Prostaglandin G(_2)</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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<tr>
<td>PGH₂</td>
<td>Prostaglandin H₂</td>
</tr>
<tr>
<td>PHS</td>
<td>Prostaglandin H Synthase</td>
</tr>
<tr>
<td>SC-560</td>
<td>5-(4-Chlorophenyl)-1-(4-methoxyphenyl)-3-trifluoromethyl pyrazole</td>
</tr>
<tr>
<td>S.D.</td>
<td>Standard Deviation</td>
</tr>
<tr>
<td>TMPD</td>
<td>N,N,N',N'-tetramethyl p-phenylenediamine</td>
</tr>
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<td>Units</td>
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<td>v/v</td>
<td>Volume/Volume</td>
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Chapter 1

General Introduction

1.1 Statement of the Research Problem

Cancer is defined as a group of diseases that are characterized by the uncontrolled proliferation and metastasis of abnormal cells (Bishop and Weinberg, 1996). These diseases continue to be the leading causes of premature death in Canadian men and women (Canadian Cancer Society, 2007). Cancer development is a multi-step process that is characterized by three main events; initiation, promotion and progression (Bishop and Weinberg, 1996).

Cancer can be initiated upon exposure to a chemical or physical agent. In most cases, enzymatic bioactivation is required in order to convert the non-toxic chemical agents into carcinogens that are capable of altering DNA (Bishop and Weinberg, 1996).

A report presented by the Canadian Cancer Society states that lung cancer is the second most frequently diagnosed cancer type in Canadians and is the cancer type that is most frequently associated with deaths (Canadian Cancer Society, 2007). Tobacco smoke is responsible for approximately 85% of the new lung cancer cases diagnosed every year (Canadian Cancer Society, 2007).

As with many other carcinogens, those present in tobacco smoke need to undergo bioactivation in order to exert their tumourigenic effects. The enzymes involved in the bioactivation of tobacco-smoke carcinogens have not all been identified. It is important to characterize these enzymes in order develop novel
chemopreventive therapies to inhibit tumour induction. Also, genetic studies could be conducted to identify those individuals who are high bioactivators of these tobacco-specific carcinogens and who could therefore be more susceptible to developing tobacco-smoke-induced lung cancer. The studies described in this thesis have been conducted in order to help identify the enzymes involved in the bioactivation of the tobacco-specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), focusing on the enzymes prostaglandin H synthase (PHS) and cytochrome P450 (CYP) 2F.

1.2 Carcinogenesis

Carcinogenesis is defined as “the conversion of normal cells responsive to homeostatic feedback mechanisms to cells capable of autonomous growth” (Bertram, 2000).

1.2.1 Carcinogenesis as a Multi-Phase Process

The first clearly described example of chemical carcinogenesis was reported in the late 18\textsuperscript{th} century by Sir Percivall Pott, who noticed a high incidence of scrotal cancer in men who worked as chimney sweeps in their youth (Bishop and Weinberg, 1996). Pott theorized that the development of scrotal cancer was a consequence of exposure to soot from incompletely burnt coal (Bishop and Weinberg, 1996). Since that discovery, advances have been made in the understanding of cancer development and it is now widely accepted that cancer progression involves three main phases; initiation, promotion and progression (Bishop and Weinberg, 1996).
1.2.1.1 Tumour Initiation

In initiation, a change is made to the cell’s DNA that stimulates a normal cell to become a cancerous one (Bishop and Weinberg, 1996). The agents that cause DNA alteration are known as carcinogens and can either be chemical in nature, such as benzo[a]pyrene (B[a]P), or physical in nature, such as UV radiation (Bertram, 2000). Initiation can also occur via spontaneous DNA damage, for example as a consequence of errors in DNA replication (Bertram, 2000).

In many cases, it is not the parent chemical compounds that drive carcinogenesis. Rather, parent compounds are converted into ultimate carcinogenic compounds via enzymatic bioactivation (Bishop and Weinberg, 1996). For example, B[a]P is bioactivated by CYP enzymes into the proximate carcinogen B[a]P-7,8-epoxide (Pitot and Dragan, 1996). This metabolic intermediate is then converted into the ultimate carcinogen, B[a]P-7,8-diol-9,10-epoxide, by the actions of CYP and PHS. This arene oxide can then bind to DNA causing damage and mutations (Parkinson, 1996).

A cell with damaged DNA is not considered to be initiated until that cell undergoes a cycle of DNA replication to convert the damaged DNA into a fixed mutation (Pitot and Dragan, 1996). However, most DNA damage is repairable and different repair mechanisms have evolved to correct the different possible types of DNA damage (Bertram, 2000). Examples of these mechanisms include base excision repair (BER), nucleotide excision repair (NER) and repair via alkyltransferases. In general, the BER pathway is employed to repair single altered nucleotides that can arise due to oxidative stress or hydrolytic
deamination or depurination of nucleotides (Norbury and Hickson, 2001). The NER pathway is generally employed to repair single nucleotides with bulky adducts or dimerized bases, among other types of damage (Pitot and Dragan, 1996). Alkyltransferases are involved in the removal and transfer of methyl or ethyl groups from guanine bases onto a cysteine acceptor on the alkyltransferase protein (Pitot and Dragan, 1996).

1.2.1.2 Tumour Promotion

Promotion is the process by which the initiated cell proliferates to form cancerous lesions (Bishop and Weinberg, 1996). Tumour promoters are themselves not mutagenic, but are capable of creating conditions that would increase the likelihood of cell proliferation into cancerous lesions (Foulds, 1958). Examples of tumour promoters include phorbol esters, compounds that are analogous to the second messenger diacylglycerol. Like diacylglycerol, phorbol esters are able to directly stimulate protein kinase C to drive cell proliferation. Promotion is considered a reversible process since the initiated cells can return to their dormant state when the promoting agent is removed (Foulds, 1958).

1.2.1.3 Tumour Progression

If the promoting agent persists, the initiated cells acquire multiple mutations in crucial genes which allow them to proliferate uncontrollably until they have formed a primary tumour (Bertram, 2000). This process is known as progression.
1.2.1.4 Tumour Metastasis

Certain cells within the primary tumour have the ability to acquire vital genetic alterations that would allow them to metastasize (Hanahan and Weinberg, 2000). These alterations include: self-sufficiency in growth signals, whereby tumour cells can generate their own growth signals, allowing them to proliferate constantly; insensitivity to antigrowth signals, whereby mutations cause certain proteins to lose their functionality, thereby leading to uncontrolled cell proliferation; evading apoptosis, where tumour cells acquire a mutation in pro-apoptotic genes thereby bypassing this process; limitless replicative potential, whereby mutations acquired allow for the increased expression of certain enzymes which cause the tumour cells to replicate infinitely; sustained angiogenesis, whereby the tumour cells sustain the growth of new blood vessels around them allowing increasing levels of oxygen and nutrients access to the cells; and tissue invasion, whereby alterations in the expression of cadherins and integrins allow cells to detach from the primary tumour and travel, via the blood, to other tissues (Hanahan and Weinberg, 2000).

1.2.2 Tobacco Smoking and Lung Cancer

Worldwide, tobacco smoking was responsible for approximately 4.2 million deaths in 2000, with over 80% of the mortalities occurring in males (World Health Organization, 2007). In Canada, cancer is reported to be the leading cause of premature death in males and females, and it is estimated that there will be 160,000 new cancer cases and 73,000 cancer deaths in Canada in 2007 (Canadian Cancer Society, 2007). Tobacco smoking is estimated to account for
85% of the new lung cancer cases seen in Canadians every year (Canadian Cancer Society, 2007). Lung cancer remains the second most frequently diagnosed cancer type, behind prostate cancer in men and breast cancer in women. However, it is the cancer type that is most frequently associated with death in both genders (Canadian Cancer Society, 2007).

The incidence rate of lung cancer in males has been declining since the mid-nineties and, today, the rate is estimated to be 68.1 cases per 100,000 males per year (Canadian Cancer Society, 2007). A corresponding decline in the male mortality rate has also been observed. Today, the mortality rate is estimated to be 60.8 deaths per 100,000 males per year (Canadian Cancer Society, 2007).

The trends in the incidence and mortality rates observed in females are opposite to the trends seen in males (Canadian Cancer Society, 2007). Since the late nineteen seventies, the incidence of lung cancer in females has continually increased, with the rate at an estimated high of 50 cases per 100,000 females per year in 2007 (Canadian Cancer Society, 2007). The mortality rate in females has also been increasing steadily since the late seventies, and is at an estimated high of 40.2 mortalities per 100,000 females per year in 2007 (Canadian Cancer Society, 2007). The trends in incidence and mortality may reflect the earlier decline in the consumption of cigarettes observed in male smokers compared to female smokers (Canadian Cancer Society, 2007).

In addition to lung cancer, tobacco smoking may be responsible for the increase in risk of several other cancer types, including bladder, ureter, kidney and liver (Hecht, 2006). Tobacco smoke contains within it over 50 different carcinogens, including the polycyclic aromatic hydrocarbon B[a]P, the aromatic
amine 4-aminobiphenyl, and the N-nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) (Hecht, 2006).

1.3 4-(MethylNitrosamino)-1-(3-pyridyl)-1-butanone (NNK)

NNK, short for nicotine-derived nitrosamine ketone, is present in substantial quantities in both unburned tobacco and tobacco smoke (Hecht, 1998). It is considered a Class 1 carcinogen by the International Agency for Research on Cancer, indicating that it is carcinogenic to humans. NNK is present in tobacco smoke at higher concentrations compared to other Class 1 carcinogens, such as B[a]P and 4-aminobiphenyl, and is able to selectively induce the formation of lung adenocarcinomas in all rodent species tested regardless of the route by which it is administered (Hecht, 1998).

1.3.1 Adenocarcinoma and NNK

Over time, the incidence of lung adenocarcinoma in Canada and the United States has increased. Adenocarcinoma has now surpassed squamous cell carcinoma as the most common form of lung cancer to arise due to tobacco smoking and several suggestions have been made to explain this trend (Hecht, 2006).

One hypothesis involves the early detection of tumours, made possible as a result of advanced diagnostic techniques that allow biopsies to be performed in the small, distal airways where adenocarcinomas normally arise (Thun et al., 1997).
The second hypothesis centres around two important changes made to the design of cigarettes. The first change involves the introduction of filters to the cigarette tips; this change was designed to decrease the levels of tar and nicotine entering the smokers’ lungs (Hoffmann et al., 1996). However, to satisfy their nicotine cravings, smokers needed to take more puffs per minute and inhale the tobacco smoke more deeply. As a consequence, carcinogens within tobacco smoke could more readily gain access to the bronchioalveolar junction and the smaller bronchi; these actions correlated with the increasing occurrence of adenocarcinomas in recent decades (Thun et al., 1997).

The second change in the design of cigarettes involves an increase in nitrate levels in tobacco blends. This increase correlated with an increase in the levels of NNK in mainstream tobacco smoke and an increase in the levels of lung adenocarcinomas (Thun et al., 1997).

1.3.2 Formation of NNK

The tertiary amine nicotine is the most active pharmacological ingredient in tobacco smoke (Kalant and Roschlau, 1998). It is a dangerous, dependence-producing compound and dependence on nicotine is the main reason for the continued use of tobacco (Hoffman and Hecht, 1985). Nicotine, on its own, is not a carcinogen but undergoes nitrosation – the addition of a nitrite group – during the curing, burning and processing of the tobacco leaf in order to be converted into the highly carcinogenic compound, NNK (Hoffman and Hecht, 1985).
1.3.3 **Metabolism of NNK**

Like many carcinogens, NNK must be bioactivated in order to exert its tumorigenic effects (Hecht, 1998). The pathway of NNK metabolism is well characterized and is illustrated in figure 1.1.

The major product of NNK metabolism is 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL). NNAL is not a detoxification product of NNK; rather, both NNK and NNAL can be bioactivated into intermediates that can bind to and modify DNA (Hecht, 1998).

Bioactivation of NNK and NNAL occurs via two processes; \( \alpha \)-methylene carbon hydroxylation and \( \alpha \)-methyl carbon hydroxylation. These processes lead to the formation of species that can methylate or pyridyloxobutylate DNA, respectively (Hecht, 1998).

In \( \alpha \)-methylene carbon hydroxylation, the methylene carbons of NNK and NNAL are converted into unstable intermediates that decompose to form methylidiazohydroxides (Hecht, 1998). These reactive species cause formation of methyl adducts in DNA (Hecht, 1998). The end-point metabolites of \( \alpha \)-methylene carbon hydroxylation are 4-oxo-4-(3-pyridyl) butyric acid (keto acid), which is formed from NNK, and 4-hydroxy-4-(3-pyridyl) butyric acid (hydroxy acid), which is formed from NNAL (Hecht, 1998).

\( \alpha \)-Hydroxylation of the methyl carbon of NNK and NNAL leads to the formation of unstable intermediates that decompose to form pyridyloxobutyl and pyridylhydroxybutyl diazohydroxides (Hecht, 1998). These reactive species form bulky adducts with DNA (Hecht, 1998). The end-point metabolites of \( \alpha \)-methyl carbon hydroxylation are 4-oxo-4-(3-pyridyl)-1-butanol (keto alcohol), which is
**Figure 1.1:** Pathway of NNK metabolism. To the right of NNK is NNAL. Indicated with ● are the reactive species that form pyridyloxobutyl adducts with DNA. Indicated with ★ are the compounds that form methyl adducts with DNA. At the top are the products of NNK and NNAL detoxification and at the bottom are the end-point metabolites of NNK and NNAL bioactivation (Bedard et al., 2002).
formed from NNK, and 4-hydroxy-4-(3-pyridyl)-1-butanol (diol), which is formed from NNAL (Hecht, 1998).

Detoxification of NNK and NNAL takes place via pyridine $N$-oxidation. In this process, the pyridine nitrogens of NNK and NNAL are oxidized and the resulting $N$-oxide products are eliminated from the body through the urine (Hecht, 1998). Detoxification of NNAL can also take place via glucuronidation, leading to the formation of a glucuronide conjugate which is also eliminated through the urine (Hecht, 1998).

1.4 Enzymes Involved in NNK Metabolism

A number of studies have attempted to identify the enzymes involved in NNK metabolism. These studies have been conducted using a variety of experimental models, including human and rodent lung and liver tissue (Lamoureux and Castonguay, 1997; Smith et al., 2003) and heterologously-expressed CYP enzymes (Smith et al., 1992; Su et al., 2000).

1.4.1 Carbonyl Reductase

NNAL is formed via carbonyl reduction of NNK (Hecht, 1998) (figure 1.1). This reaction involves the reduction of the aldehyde group on NNK to an alcohol group and is catalyzed by carbonyl reductase. The reaction is reversible, but favours NNAL formation (Hecht, 1998). One enzyme that has been demonstrated to catalyze this reaction is microsomal 11β-hydroxysteroid dehydrogenase type 1 (Finckh et al., 2001). As indicated in figure 1.1, NNAL is
an important metabolite as it too can be bioactivated into species that bind to DNA.

1.4.2  **Cytochrome P450 (CYP)**

Extensive evidence exists to support the role of rodent and human CYP enzymes in NNK biotransformation (Smith et al., 1992; Smith et al., 1995; Smith et al., 1999). However, the identities and levels of CYPs expressed, as well as CYP contributions to pulmonary NNK biotransformation can vary between species.

1.4.2.1  **Rodents**

In rats and mice, evidence has been gathered implicating CYPs 1A2, 2A1, 2B1 and 3A in NNK bioactivation in both lung and liver tissues (reviewed by Hecht, 1998).

1.4.2.2  **Humans**

To date, a number of isozymes have been implicated in human pulmonary NNK biotransformation, including CYPs 2E1, 2A6 and 2A13. A study performed with human lung microsomes demonstrated that the addition of antibodies against CYP2E1 resulted in a decreased level of NNK end-point metabolites (Smith et al., 2003). However, this decrease was not seen in all the microsomes suggesting that CYP2E1 can metabolize NNK only in certain individuals (Smith et al., 2003).
Another enzyme involved in pulmonary NNK metabolism is CYP2A13 (Su et al., 2000). This enzyme is predominantly expressed in the respiratory tract and has been detected in the human lung, trachea and nasal mucosa (Su et al., 2000). Heterologously-expressed CYP2A13 can catalyze the formation of keto acid and keto alcohol from NNK (Su et al., 2000).

The contribution of CYP2A6 to NNK α-carbon hydroxylation was first seen when NNK was incubated with hepatoma cells expressing CYP2A6 (Smith et al., 1992). This finding was verified when it was demonstrated that the addition of CYP2A6 antibodies to human lung microsomes significantly inhibited the formation of metabolites reflective of α-carbon hydroxylation in all patients tested (Smith et al., 1995). A later study demonstrated that 8-methoxypsoralen, a CYP2A6 chemical inhibitor, decreased the level of NNK end-point metabolites in lung microsomes from five of the seven patients tested (Smith et al., 2003). 8-Methoxypsoralen is also an inhibitor of CYP2A13 (von Weymarn et al., 2005; Nakajima et al., 2006) and therefore it is possible that the decrease in NNK end-point metabolites occurred as a result of inhibition of both CYP2A6 and 2A13 (Smith et al., 2003).

An additional CYP subfamily that could contribute to NNK biotransformation is CYP2F. This subfamily is unusual because it consists of only a single functional member in each of the species examined; 2F1 in humans, 2F2 in mice, 2F3 in goat and 2F4 in rats (Chen et al., 2002). These enzymes show a nucleic acid and amino acid sequence identity of over 80% (Chen et al., 2002). The CYP2F genes are selectively expressed in lung, with little or no expression in liver (Chen et al., 2002). CYP2F enzymes have been implicated in
the bioactivation of other lung toxicants, including 3-methylindole (3-MI) (Lanza et al., 1999), naphthalene (Lanza et al., 1999) and styrene (Nakajima et al., 1994).

In addition to α-carbon hydroxylation, it appears that CYPs play a role in pyridine N-oxidation (Hecht, 1998). One study demonstrated a correlation between CYP3A4 activity and the formation of NNK-N-oxide in human hepatocytes (Staretz et al., 1997). In addition, the non-selective CYP inhibitors proadifen hydrochloride and 1-aminobenzotriazole caused a decrease in the level of N-oxidation in four of the five human lung microsomes tested (Smith et al., 2003).

1.4.3 Peroxidases

While many studies have demonstrated that CYPs participate actively in NNK metabolism, they may not be the only enzymes involved in this process. Another group of enzymes that may play a role in NNK metabolism are peroxidases (Smith et al., 1995).

Peroxidases are a family of heme-containing enzymes that can catalyze the oxidation of a substrate by a peroxide (O'Brien, 2000). Members of the peroxidase family include lipoxygenase (LOX) and PHS (O'Brien, 2000). These enzymes are capable of oxidizing a variety of xenobiotics, and their possible involvement in NNK biotransformation was first demonstrated when the addition of indomethacin and nordihydroguaiaretic acid, inhibitors of PHS and LOX respectively, to human lung microsomes resulted in a decreased level of NNK metabolites (Smith et al., 1995).
1.4.3.1 **Lipoxygenase (LOX)**

LOX is widely distributed in mammalian tissue and catalyzes the addition of a single molecule of oxygen to arachidonic acid to form leukotrienes (Gale and Egan, 1984). Although previously reported to be capable of metabolizing NNK, LOX was eliminated as an NNK metabolizing enzyme when it was demonstrated that incubation of NNK with LOX purified from human lung failed to result in formation of NNK metabolites (Bedard et al., 2002). Likewise, the model enzyme, soybean LOX, failed to catalyze formation of NNK end-point metabolites (Bedard et al., 2002).

1.4.3.2 **Prostaglandin H Synthase (PHS)**

PHS is present in almost every mammalian tissue including neural tissue, pulmonary tissue and bladder tissue (Eling et al., 1990). It exists as at least two isoforms; PHS-1 and PHS-2. A third isoform, PHS-3, has been identified in the canine cerebral cortex (Simmons, 2003). This isoform is a variant of PHS-1; under normal circumstances, intron 1 is spliced out of PHS-1. However, this intron is retained in PHS-3 resulting in an extra 30 amino acids being added. This could lead to alterations in protein folding and enzyme activity (Schwab et al., 2003). It is still unclear if this isoform exists in humans; a sequencing study performed on the human PHS-1 gene showed that human intron 1 contained one extra nucleotide compared to canine intron 1 (Berenbaum, 2004). This extra nucleotide could cause a frameshift in the nucleic acid sequence which could in turn result in a human PHS-3 isoform with a different conformation compared to the canine PHS-3 isoform (Berenbaum, 2004).
In general, PHS-1 is considered to be the constitutively expressed isoform, while PHS-2 expression is considered to be inducible. However, PHS-1 is inducible under inflammatory conditions in the kidney (Hartner et al., 2000; Graupera et al., 2005) and PHS-2 expression is constitutive in the brain, kidney and blood vessels (Warner and Mitchell, 2004). In general, PHS-1 is involved in the formation of prostaglandins that take part in cellular housekeeping functions, while PHS-2 plays a role in the inflammatory response resulting from tissue damage (Simmons et al., 2004).

1.4.3.2.1 Structure of PHS

Both PHS-1 and PHS-2 exist as homodimers with each monomer having a mass of approximately 70 kilodaltons (Kurumbail et al., 2001). The two isoforms are encoded by distinct genes located on different chromosomes, but share a high degree of amino acid sequence similarity and structural homology (Bingham et al., 2006) (figure 1.2(a)). In addition, both isoforms have three distinct domains: the dimerization domain, the membrane binding domain and the catalytic domain (Simmons et al., 2004) (figure 1.2(b)). The dimerization domain holds the subunits together via hydrogen bonds, salt bridges and hydrophobic interactions (Simmons et al., 2004). The membrane binding domain anchors the protein to the lumen of the endoplasmic reticulum and nuclear envelope via hydrophobic interactions. The catalytic domain comprises most of the protein and contains two distinct active sites; the cyclooxygenase active site, located at the end of a long, narrow hydrophobic channel, and the peroxidase active site, located on the upper surface of the protein.
Figure 1.2: Crystallographic structures of ovine PHS-1 and murine PHS-2.

(a) Structural homology of ovine PHS-1 (blue) and murine PHS-2 (green). The membrane binding domains are indicated in blue and purple (Bingham et al., 2006).

(b) Three domains of ovine PHS-1 (left) and murine PHS-2 (right): dimerization (green), membrane binding (yellow) and catalytic (blue), can all be seen. The heme group (red) is also indicated (Simmons et al., 2004).
(Simmons et al., 2004). The peroxidase active site is also the region in which heme is bound. An important difference between PHS-1 and PHS-2 is in the structure of their active sites; in PHS-2, the amino acid isoleucine is replaced by valine resulting in a larger, more open cyclooxygenase active site compared to PHS-1 (Simmons et al., 2004). This change is important as it allows for substrate and inhibitor specificity with PHS-2. These two active sites play an important role in the formation of prostaglandins, as discussed below.

1.4.3.2.2 Formation of Prostaglandins

Prostaglandins (PG) are potent lipid mediators that are found in virtually all tissues and organs. They are derived enzymatically from arachidonic acid through the combined action of the cyclooxygenase and peroxidase activities of PHS-1 and PHS-2 (Simmons et al., 2004) (figure 1.3).

In the cyclooxygenase reaction, PHS catalyzes the addition of two molecules of oxygen to one molecule of arachidonic acid, leading to the formation of the cyclic endoperoxide hydroperoxide, prostaglandin G₂ (PGG₂) (Simmons et al., 2004). Briefly, the mechanism involves the removal of the hydrogen atom at carbon-13 of arachidonic acid to yield an arachidonyl radical. A molecule of oxygen is then added to the radical to form an endoperoxide bridge between carbon-9 and carbon-11. A bicyclic structure is then formed when stereospecific rearrangement generates a bond between carbon-8 and carbon-12. A second molecule of oxygen is then added at carbon-15 yielding the hydroperoxide PGG₂ (O'Brien, 2000).
Figure 1.3: Formation of prostaglandins. In the cyclooxygenase reaction, PHS catalyzes the addition of two oxygen molecules to arachidonic acid to form PGG$_2$. In the peroxidase reaction, PHS catalyzes the reduction of PGG$_2$ to PGH$_2$. During the peroxidase reaction, xenobiotic bioactivation can occur when an oxygen atom is transferred from PGG$_2$ to the xenobiotic (X). This process is known as cooxidation.
The peroxidase activity requires the heme group and, in this reaction, the hydroperoxide PGG$_2$ is reduced to the corresponding alcohol, prostaglandin H$_2$ (PGH$_2$) (Eling et al., 1990). The heme group exists in the Fe(III) state at rest. In the presence of a peroxide, Fe(III) is oxidized to compound I, an intermediate in the Fe(V) state. Compound I is then reduced to compound II, which is in the Fe(IV) state. This is further reduced to the resting enzyme containing Fe(III) (figure 1.4). The reduction of compound I to resting state occurs at the same time as PGG$_2$ is reduced to PGH$_2$ (Eling et al., 1990). This reaction requires a cosubstrate capable of electron donation, examples of which include phenols, aromatic amines and certain xenobiotics (Eling et al., 1990).

PGH$_2$ can then be converted into other prostaglandins including thromboxanes, prostacyclins and prostaglandins E$_2$ and F$_{2\alpha}$ (Simmons et al., 2004).

### 1.4.3.2.3 PHS-Mediated Xenobiotic Biotransformation

PHS-catalyzed xenobiotic biotransformation occurs via cooxidation, a process that couples the oxidation of the xenobiotic with the reduction of lipid hydroperoxides (Parkinson, 1996). Cooxidation can take place either during the cyclooxygenase reaction or during the peroxidase reaction.

Biotransformation by cyclooxygenase activity of PHS-1 and PHS-2 is mediated via the leakage of peroxyl radicals (LOO$^\cdot$) which are formed upon the addition of one molecule of oxygen to arachidonic acid (Reed, 1987).
Figure 1.4: Proposed mechanism for the peroxidase activity of PHS. Fe(III) is oxidized by hydrogen peroxide (H$_2$O$_2$) into Compound I (which contains Fe(V)). Compound I is then reduced to Compound II (which contains Fe(IV)) which is then further reduced to resting state. The reduction of compound I to resting state can take place in the presence of xenobiotics (X). When these xenobiotics donate their electrons to heme, they can potentially form free radical species that interact with DNA (Eling et al., 1990).
Xenobiotic biotransformation by the peroxidase activity of PHS-1 and PHS-2 can occur via the direct transfer of the peroxide oxygen of PGG2 to the xenobiotic (figure 1.3). Two lung carcinogens that can undergo cooxidation are B[a]P (Panthananickal and Marnett, 1981; Marnett et al., 1977; Marnett and Reed, 1979), and aflatoxin B1 (Battista and Marnett, 1985; Liu et al., 1990).

B[a]P can be bioactivated by PHS into quinone intermediates during the oxidation of arachidonic acid (Eling et al., 1990). These intermediates are considered to be only moderately cytotoxic and do not bind covalently to DNA (Marnett et al., 1977; Marnett and Reed, 1979). However, studies have shown that B[a]P-7,8-diol (the metabolized form of B[a]P) can be converted by PHS into reactive electrophilic derivatives that are capable of binding to DNA (Panthananickal and Marnett, 1981; Marnett et al., 1978; Wiese et al., 2001).

It has also been demonstrated that aflatoxin B1, in the presence of arachidonic acid and purified PHS, can be converted into an epoxide that binds to DNA (Battista and Marnett, 1985; Liu et al., 1990).

In addition, recent work has demonstrated the ability of ovine and human PHS-1 and PHS-2 to bioactivate 4-aminobiphenyl into DNA binding species (Wiese et al., 2001). Like NNK, 4-aminobiphenyl is a class I lung carcinogen that is present in tobacco smoke, although it is found at lower levels than is NNK (Hecht, 2006).

Since PHS-1 and PHS-2 are capable of metabolizing the pulmonary carcinogens mentioned above, it is possible that they are also able to biotransform NNK.
1.5 **Research Hypotheses and Objectives**

According to the World Health Organization, there are almost one billion male smokers and 250 million female smokers in the world. The global consumption of cigarettes is slowly declining as more people are recognizing the risks and consequences associated with tobacco smoke. However, due to population expansion, the World Health Organization estimates that tobacco smoking will still be responsible for ten million deaths worldwide by 2030.

NNK is widely recognized to be an important pulmonary carcinogen in tobacco smoke. It is a selective inducer of lung adenocarcinoma in all rodent species tested and is thought to be responsible for the increase in the incidences of adenocarcinoma in Canadian men and women. It is important to elucidate the enzymes responsible for pulmonary NNK biotransformation in order to design treatment plans involving the inhibition or enhancement of enzyme activity. Unfortunately, many of the enzymes that play a role in NNK biotransformation have yet to be elucidated. Previous work has demonstrated that cytochrome P450s are important metabolizers of NNK but there is also evidence that other enzymes could be involved. Some of the enzymes that may contribute to NNK biotransformation are prostaglandin H synthase and cytochrome P450 2F.

To date, only one study has reported a role for prostaglandin H synthase in the biotransformation of NNK. However, in that study, the metabolite levels detected were far higher than those detected by our laboratory in the past, and the conditions and reaction components that were utilized were more favourable of NADPH-dependent processes than of arachidonic acid-dependent processes.
To the best of our knowledge, very little work has been done in determining whether or not CYP2F is involved in NNK biotransformation. One study reported bioactivation in the presence of hepatoma cells expressing CYP2F1. However, the NNK concentrations were higher than those normally used and concern has been expressed about those cells containing low levels of CYP2F1 (Dr. Garold S. Yost, personal communication). CYP2F exists as a single isoform, and is predominantly expressed in the lung, with little or no expression in the liver. It would therefore be of interest to establish whether or not CYP2F contributes to the pulmonary biotransformation of NNK.

Based on the background information, two research hypotheses were formulated.

**Hypothesis 1:** Prostaglandin H synthase enzymes are capable of biotransforming NNK.

**Objective 1(a):** To assess the effects of a general PHS inhibitor and isoform-selective PHS inhibitors on NNK bioactivation and detoxification in human lung microsomes.

**Objective 1(b):** To confirm that the cyclooxygenase and peroxidase activities of commercially available PHS enzymes are functionally active.

**Objective 1(c):** To assess the ability of commercially available purified PHS enzymes to metabolize NNK according to previously published conditions.

**Objective 1(d):** To assess the ability of commercially available purified PHS enzymes to metabolize NNK in the presence of cofactors and conditions that are optimal for PHS activity.
Hypothesis 2: CYP2F enzymes are capable of biotransforming NNK.

Objective 2(a): To assess the effects of a CYP2F inhibitor on NNK biotransformation in human lung microsomes.

Objective 2(b): To assess the ability of purified goat recombinant CYP2F3 to biotransform NNK.
Chapter 2

Investigation of the Biotransformation of 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) by Prostaglandin H Synthase (PHS)

2.1 Introduction

In Canada, lung cancer is the second most frequently diagnosed cancer type in males and females, and is the cancer type that is most frequently associated with death in both genders (Canadian Cancer Society, 2007). According to a report by the Canadian Cancer Society, tobacco smoking is responsible for 85% of the new lung cancer cases seen every year (Canadian Cancer Society, 2007).

Tobacco smoke contains within it over 50 different carcinogens, one of the most important being 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) (Hecht, 2006). NNK, short for nicotine-derived nitrosamino ketone, has been categorized as a Class 1 carcinogen by the International Agency for Research on Cancer, indicating that it is carcinogenic in humans (Hecht, 2006). It is present in tobacco smoke at higher amounts compared to other Class 1 carcinogens such as B[a]P and 4-aminobiphenyl, and has been demonstrated to be a potent and selective inducer of lung adenocarcinoma in all rodent species tested (Hecht, 1998).

As with many chemical carcinogens, NNK needs to undergo enzymatic bioactivation in order to exert its tumourigenic effects. The major metabolite of
NNK is 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) (figure 1.1). NNAL is not a detoxification product of NNK; rather, both NNK and NNAL are bioactivated into species that can bind to and alter DNA (Hecht, 1998). Bioactivation of NNK and NNAL occurs via α-carbon hydroxylation, resulting in the formation of reactive species that are capable of methylating and pyridyloxobutylating DNA. The end-point metabolites of NNK bioactivation are keto alcohol and keto acid, and those of NNAL bioactivation are hydroxy acid and diol. Detoxification of NNK and NNAL takes place via pyridine N-oxidation, resulting in the formation of NNK N-oxide and NNAL N-oxide which are excreted from the body through urine (Hecht, 1998).

In the past, several studies have been conducted in order to characterize the enzymes responsible for NNK biotransformation. These studies have implicated CYPs as the major enzyme family responsible for metabolism; however, the studies have also demonstrated a role for other enzyme families in this process (Smith et al., 1995; Su et al., 2000; Smith et al., 2003). One such enzyme family could be peroxidases.

Peroxidases are a group of heme-containing enzymes that can catalyze the oxidation of a substrate by a peroxide (O'Brien, 2000). Two members of this family are PHS-1 and PHS-2 (Eling and Curtis, 1992). PHS-1 is generally recognised as the constitutively expressed enzyme; however, several studies have demonstrated its induction in the kidney under inflammatory conditions (Hartner et al., 2000; Graupera et al., 2005). PHS-2 is generally recognized to be the inducible enzyme, but its expression has been shown to be constitutive in the brain, kidney, and blood vessels (Warner and Mitchell, 2004).
The main function of the PHS isoforms is in the production of prostaglandins, lipid mediators that are present in almost every tissue (Simmons et al., 2004). Prostaglandins are formed via a sequence of two reactions – the cyclooxygenase reaction and the peroxidase reaction (Eling and Curtis, 1992) (figure 1.3). In the cyclooxygenase reaction, PHS catalyses the addition of two molecules of oxygen to a molecule of arachidonic acid to form PGG$_2$. In the peroxidase reaction, PHS catalyses the reduction of PGG$_2$ to PGH$_2$ (Eling and Curtis, 1992).

PHS-catalyzed xenobiotic biotransformation takes place via cooxidation, a process that couples the oxidation of the xenobiotic with the reduction of lipid hydroperoxides (Parkinson, 2001). This can occur either by the cyclooxygenase or peroxidase activities of PHS. Biotransformation by the cyclooxygenase activity is mediated via the leakage of peroxyl radicals (LOO‘) which are formed upon the addition of one molecule of oxygen to arachidonic acid (Reed, 1987). Biotransformation by the peroxidase activity can occur via the direct transfer of the peroxide oxygen to the xenobiotic (Parkinson, 2001). Two lung carcinogens that are bioactivated by PHS are B[a]P (Panthananickal and Marnett, 1981; Marnett et al., 1977; Marnett and Reed, 1979), and aflatoxin B$_1$ (Battista and Marnett, 1985; Liu et al., 1990).

The ability of PHS to biotransform NNK using purified ovine enzymes and murine lung microsomes has been reported (Rioux and Castonguay, 1998b) however, there were several problems with the study. First, the experiments performed utilized conditions and cofactors more suited for NADPH-dependent processes (e.g. CYP reactions) rather than arachidonic acid-dependent
processes. Also, the experiments were performed in mouse lung microsomes; PHS-mediated NNK biotransformation in human lung microsomes was not examined. Finally, the metabolite levels detected in Rioux and Castonguay’s study were much higher than those detected in similar studies performed in our laboratory and in others (Rioux and Castonguay, 1998b). The purpose of the present study was to determine whether PHS enzymes are involved in NNK biotransformation in human lung microsomes.

2.2 Materials and Methods

2.2.1 Chemicals

Chemicals were obtained from suppliers as follows: [5-\(^3\)H]NNK (2.5-11.0 Ci/mmol; >98% pure) from Moravek Biochemicals (Brea, CA), NNK, NNAL, NNK-N-oxide, NNAL-N-oxide, hydroxy acid, keto acid, diol, and keto alcohol from Toronto Research Chemicals (North York, ON), Uniscint BD Radioflow Scintillation Cocktail from National Diagnostics (Atlanta, GA), arachidonic acid (sodium salt; ~99% pure), hematin (porcine), glucose-6-phosphate dehydrogenase, N, N, N', N'-tetramethyl p-phenylenediamine, indomethacin, 5-(4-chlorophenyl)-1-(4-methoxyphenyl)-3-trifluoromethyl pyrazole and N-[2-(cyclohexyloxy)-4-nitrophenyl]methanesulfonamide from Sigma-Aldrich (St. Louis, MO) and purified ovine PHS-1 and PHS-2 from Cayman Chemical Company (Ann Arbor, MI). All other chemicals were reagent grade and obtained from common commercial suppliers.
2.2.2 Procurement of Lung Tissue

The Queen’s University Ethics Review Committee for Research on Human Subjects approved the use of human lung tissue for these studies. Following informed consent, samples of peripheral lung tissue devoid of macroscopically visible tumours were obtained from patients undergoing clinically indicated lobectomies at the Kingston General Hospital. Each tissue was cut into 1 cm³ pieces and stored at -80°C until required for microsome preparation. Patients were characterized according to age, gender, surgical diagnosis, occupational exposure to carcinogens, drug treatment one month prior to surgery and smoking history. Patients were classed as former smokers if they ceased smoking more than two months prior to surgery; this time period is considered to be adequate for return of biotransformation enzyme activities to normal levels following exposure to inducing agents in cigarette smoke (Smith et al., 1999).

2.2.3 Preparation of Human Lung Microsomes

Lung tissue was removed from -80°C storage, chopped and homogenized with a Polytron homogenizer (Brinkman Instruments, Rexdale, ON). The homogenised tissue was centrifuged in a model J2-21 centrifuge (Beckman-Coulter Inc., Fullerton, CA) at 10,000xg for 20 minutes. The pellets, containing exogenous tissue and fibrous lung components, were discarded while the supernatant, containing the microsomes and cytosol, was collected. The supernatant was centrifuged in a Beckman Optima™ XL-100K Ultracentrifuge (Beckman-Coulter Inc., Fullerton, CA) at 100,000xg for one hour at 4°C. The pellets were re-suspended in 0.1 M potassium phosphate buffer (pH 7.4) plus
1.15% KCl and centrifuged again at 40,000 rpm for one hour at 4°C. The final pellet was re-suspended in buffer, homogenized, aliquoted into cryovials and snap frozen in liquid N₂. The vials containing the microsomes were stored at -80°C until required for experimentation. Microsomal protein levels were determined according to the Lowry method using bovine serum albumin as the standard (Lowry et al., 1951).

2.2.4 Purification of [5-³H]NNK

Prior to use, the purity of radiolabelled [5-³H]NNK was determined. When [5-³H]NNK purity was less than 98%, the isotope was re-purified before being used in experiments. Re-purification occurred by normal-phase gradient high performance liquid chromatography (HPLC). Briefly, 100 μL of the original [5-³H]NNK solution was injected onto a Zorbax 5 micron silica column (VWR International, Mississauga, ON) and the absorbance of [5-³H]NNK was monitored at 254 nm (LabAlliance model 500 UV detector, State College, PA). The elution program delivered solvent A (hexane: chloroform, 70:30) through the column for 5 minutes followed by a linear gradient to 7% solvent B (methanol) in 28 minutes. The solvents were delivered at 1.0 mL/minute. [5-³H]NNK eluted between 36 and 41 minutes. The mobile phase was removed under N₂ gas, and [5-³H]NNK was re-suspended in HPLC grade water.
2.2.5 Incubations with [5-3H]NNK

2.2.5.1 Human lung microsomes

Human lung microsomes (1.0 mg/mL) were incubated with 4.2 µM [5-3H]NNK in a total reaction volume of 1.0 mL under NADPH-dependent and non-NADPH-dependent conditions. The NADPH-generating system consisted of 5.0 mM glucose-6-phosphate, 2.0 U glucose-6-phosphate dehydrogenase and 1.25 mM NADP. The incubations also contained 0.10 M potassium phosphate buffer (pH 7.4) plus 1.15% KCl, 1.0 mM EDTA and 3.0 mM MgCl₂.

In the absence of the NADPH-generating system, the incubations contained 0.10 M potassium phosphate buffer (pH 7.4) with 1.15% KCl, 0.3 mM arachidonic acid and 1.0 µM hematin.

Following a pre-incubation for 15 minutes at 37°C, reactions were initiated by addition of microsomes. The samples were incubated for 15 minutes at 37°C in a Dubnoff metabolic shaker (Precision Scientific, Chicago, IL), and were terminated by addition of 300 µL each of 25% zinc sulphate and saturated aqueous barium hydroxide. The incubates were centrifuged at 2500xg and the supernatants were frozen in liquid N₂ and stored at -80°C until analysis.

Incubations were also performed in the presence of chemical inhibitors, specifically the general PHS inhibitor indomethacin (10 µM), the PHS-1 selective inhibitor SC-560 (100 nM) and the PHS-2 selective inhibitor NS-398 (10 µM). Microsomes were pre-incubated with the inhibitors at 37°C for 15 minutes and the reactions were carried out as previously described. All reactions were performed in duplicate.
2.2.5.2 Purified PHS

Reactions were performed according to a previously outlined methodology (Rioux and Castonguay, 1998b). Incubations contained 10 μM [5-³H]NNK, 1000 U each of purified PHS-1 or purified PHS-2, 0.10 M sodium phosphate buffer (pH 7.4), 1.0 mM EDTA and 3.0 mM MgCl₂ for a total reaction volume of 800 μL. The NADPH-generating system consisted of 5.0 mM glucose-6-phosphate, 1.52 U glucose-6-phosphate dehydrogenase and 1.0 mM NADP. Reactions were also performed in the presence of 100 μM arachidonic acid. Following a pre-incubation for 10 minutes at 37°C, reactions were initiated by addition of either purified PHS-1 or PHS-2. The samples were incubated at 37°C for 30 minutes and were stopped by addition of 300 μL each of 25% zinc sulphate and saturated aqueous barium hydroxide. The samples were centrifuged at 14,000xg for 5 minutes and were frozen in liquid N₂ and stored at -80°C until analysis.

Under non NADPH-dependent conditions, the reactions consisted of 10 μM [5-³H]NNK, 1000 U each of purified PHS-1 or PHS-2, 0.10 M potassium phosphate buffer (pH 7.4) and 1.0 μM hematin. The reactions were initiated by addition of 100 μM arachidonic acid and incubated for 30 minutes at 37°C. The reactions were terminated via the addition of 300 μL each of 25% zinc sulphate and saturated aqueous barium hydroxide. Samples were centrifuged at 14,000xg for 5 minutes and were frozen in liquid N₂ and stored at -80°C until analysis.
2.2.5.3 Hamster Liver Microsomes

As a positive control for NNK biotransformation and NNK metabolite detection, hamster liver microsomes were incubated with 10 μM [5-3H]NNK as described by Lamoureux and Castonguay (1997). Briefly, the reactions contained 5.0 mM glucose-6-phosphate, 1.52 U glucose-6-phosphate dehydrogenase, 1.0 mM NADP, 1.0 mM EDTA, 3.0 mM MgCl₂ and 0.10 M sodium phosphate buffer (pH 7.4) in a total volume of 800 μL. Following a pre-incubation at 37°C for 10 minutes, reactions were initiated by addition of 0.4 mg/mL microsomal protein and were incubated at 37°C. Reactions were stopped after 30 minutes via the addition of 300 μL each of 25% zinc sulphate and saturated aqueous barium hydroxide and centrifuged at 14,000 xg for 5 minutes. The supernatants were then frozen in liquid N₂ and stored at -80°C until analysis. Control incubations contained boiled enzymes. Reactions were also performed in the absence of the NADPH-generating system to confirm NADPH involvement in metabolite formation.

2.2.6 Cyclooxygenase and Peroxidase Activities of PHS

To verify that the cyclooxygenase and peroxidase activities of purified PHS-1 and PHS-2 were functional, two experiments were performed. In the first, the cyclooxygenase activity of PHS-1 and PHS-2 was assessed by the protocol of Kalgutkar et al. (1996) with some modifications. The rate of oxygen consumption was observed using a model 5300 biological oxygen monitor and a model 5301 Clark-type polarographic oxygen electrode (Yellow Springs...
Instrument Co. Inc., Yellow Springs, OH). The total volume of each reaction was 2.6 mL and consisted of 100 U of purified ovine PHS-1 or PHS-2, 0.1 M Tris-HCl buffer (pH 8; containing 5.0 mM EDTA and 2.0 mM phenol) and 1.0 µM hematin. The reaction was initiated by the addition of 100 µM arachidonic acid and maintained at 37°C for 5 minutes. One unit of cyclooxygenase activity catalysed the consumption of 1 nmole of oxygen per minute.

The peroxidase activity (figure 2.1) was assessed by the method of Maccarrone et al. (1998) with some modifications. Briefly, the reactions contained 0.1 M potassium phosphate buffer (pH 7.9), 1.0 µM hematin, 100 µM arachidonic acid and 170 µM TMPD for a total reaction volume of 800 μL. The reactions were initiated by the addition of 100 or 1000 U of purified ovine PHS-1 or PHS-2 and were incubated at 37°C for 15 minutes. The increase in absorbance of oxidized TMPD was measured on a Cary 100 Bio UV-Visible Spectrophotometer (Varian Inc., Scientific Instruments, Palo Alto, CA) at 611 nm.

2.2.7 Measurement of NNK Metabolism

Metabolites were quantified by reverse-phase HPLC with radiometric detection. The filtered reaction mixture (1.0 mL) was put onto a 10 μm Bondapak C_{18} column (3.9 x 300 mm; Waters, Milford, MA). The elution program delivered solvent A (0.06 M sodium acetate, pH 6.0) for 10 minutes followed by a linear gradient to 60% solvent B (methanol: water, 1:1, v/v) for 70 minutes at a rate of 1 mL/minute. [5-\textsuperscript{3}H]NNK and its metabolites were quantified using an Insus Systems Inc. β-ram radioflow detector (Tampa, FL) and NNK
Figure 2.1: Oxidation of TMPD by the peroxidase activity of PHS-1 and PHS-2. TMPD is oxidized to a highly coloured compound that absorbs light at 611 nm.
metabolite formation was expressed as a percentage of the total radioactivity recovered from [5-\(^3\)H]NNK and all its metabolites. To determine the retention times of NNK and its metabolites, reference standards were detected with a UV detector at 254 nm.

2.2.8 Data Analysis

For NNK biotransformation, individual metabolite results represent the mean of duplicate incubations. When microsomal data were grouped, the data are presented as means ± standard deviations (S.D.). Statistically significant differences in the grouped microsomal data following inhibitor treatments were determined by repeated measures analysis of variance (ANOVA) followed by the Tukey-Kramer post-hoc test using Graphpad Prism 4. When heterogeneity of variance was revealed via Cochran’s test, a positive integer (k=1) was added to each relevant data point (since some points were equal to zero) and a log transformation was performed on the results prior to conducting ANOVA. If homogeneity of variance was not achieved with the transformed data, the Friedman non parametric test was used (Zivin and Bartko, 1976). All other statistically significant differences were determined by Student’s paired t-test. P<0.05 was considered statistically significant in all cases.
2.3 Results

2.3.1 Patient Demographics

Microsomes were prepared from sections of lung tissue obtained from four patients (three males and one female), aged 64.8 ± 13.4 years, and were used to assess the effects of arachidonic acid and PHS enzyme inhibitors on NNK metabolism (table 2.1). Based on self-reported smoking histories, three individuals were former smokers and one was a current smoker. Information regarding the smoking history of patient 178 is incomplete since the number of years that he smoked was not reported. In addition, the patient verified that he was not taking any medication except for vitamins. All four patients were diagnosed with lung adenocarcinoma. Racial demographics were not available for any of the patients examined; however, the population in the area of the Kingston General Hospital is largely caucasian.

2.3.2 [5-3H]NNK Metabolism in Human Lung Microsomes

2.3.2.1 Individual Patient Results

NNK biotransformation was assessed both in the presence of an NADPH-generating system and in the presence of arachidonic acid and hematin, two cofactors that are required for PHS-mediated biotransformation.

In the presence of the NADPH-generating system, NNK metabolism resulted in the formation of measurable amounts of keto acid, diol, keto alcohol, NNAL-N-oxide, NNK-N-oxide and NNAL. The only metabolite that could not be
### Table 2.1  Patient demographics

<table>
<thead>
<tr>
<th>Patient #</th>
<th>Age</th>
<th>Sex</th>
<th>Smoking History</th>
<th>Diagnosis Leading to Surgery</th>
<th>Drug Tx(^d) 1 Month Prior</th>
<th>Possible Occupational Exposure to Carcinogens</th>
</tr>
</thead>
<tbody>
<tr>
<td>137(^a)</td>
<td>45</td>
<td>M(^b)</td>
<td>Current 49.5 pack years(^c)</td>
<td>Adenocarcinoma</td>
<td>Diazepam (Valium), Fluvoxamine (Luvox), Warfarin (Coumadin)</td>
<td>None</td>
</tr>
<tr>
<td>177</td>
<td>70</td>
<td>M</td>
<td>Former (quit 1 year ago) 80 pack years</td>
<td>Adenocarcinoma</td>
<td>Enalapril (Vasotec), Diltiazem (Cardizem), Hydrochlorothiazide (Hydrodiuril), Atorvastatin (Lipitor), Terazosin (Hytrin), Aspirin</td>
<td>None</td>
</tr>
<tr>
<td>178</td>
<td>69</td>
<td>M</td>
<td>Former (quit 30 years ago) Pack years not available</td>
<td>Adenocarcinoma</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>179</td>
<td>75</td>
<td>F</td>
<td>Former (quit &gt;2 months ago) 50 pack years</td>
<td>Adenocarcinoma</td>
<td>Felodipine (Plendil), Ramipril (Altace), Atenolol (Tenormin)</td>
<td>None</td>
</tr>
</tbody>
</table>

\(^a\)Patients are assigned numbers for confidentiality

\(^b\)Abbreviations: M = male; F = female

\(^c\)Pack Years: number of packs smoked per day times the number of years (1 pack year = 1 pack a day for 1 year)

\(^d\)Generic Drug Name (proprietary name in brackets)
consistently detected was hydroxy acid as its levels were below the lower limit of detection (0.0005% (mg protein)\(^{-1}\) minute\(^{-1}\)). The major NNK metabolite formed was NNAL and its levels ranged from 0.227 to 0.753% (mg protein)\(^{-1}\) minute\(^{-1}\) (figure 2.2(a)). Total NNK bioactivation (represented by the sum of the four \(\alpha\)-carbon hydroxylation end-point metabolites) ranged from 0.00300 to 0.0153% (mg protein)\(^{-1}\) minute\(^{-1}\) (figure 2.2(b)) and total \(N\)-oxidation (represented by the sum of NNAL-\(N\)-oxide and NNK-\(N\)-oxide) ranged from 0.000667 to 0.00467% (mg protein)\(^{-1}\) minute\(^{-1}\) (figure 2.2(c)).

When the NADPH-generating system was replaced with arachidonic acid, less microsomal NNK metabolite formation was observed. Once again, NNAL was the major metabolite detected in all microsomes and its levels ranged from 0.000333 to 0.006% (mg protein)\(^{-1}\) minute\(^{-1}\) (figure 2.2(a)). Total NNK bioactivation ranged from 0.00250 to 0.00600% (mg protein)\(^{-1}\) minute\(^{-1}\) (figure 2.2(b)), with keto acid and keto alcohol being the only \(\alpha\)-carbon hydroxylation metabolites consistently detected (results for individual metabolites not shown). Levels of hydroxy acid and diol were below the lower limit of detection (results not shown). Metabolites reflective of \(N\)-oxidation were only detected in microsomes from patients 8M and 3J; total \(N\)-oxidation was 0.00167% (mg protein)\(^{-1}\) minute\(^{-1}\) in 8M and 0.00100% (mg protein)\(^{-1}\) minute\(^{-1}\) in 3J (figure 2.2(c)).

2.3.2.2 Grouped Patient Results

When the specific \(\alpha\)-carbon hydroxylation end-point metabolites were examined, levels of keto acid (reflective of \(\alpha\)-methylene carbon hydroxylation) were significantly higher than those of keto alcohol or diol (reflective of \(\alpha\)-methyl
Figure 2.2: Metabolism of [5-³H]NNK in the presence of the NADPH-generating system and the in the presence of arachidonic acid in individual human lung microsomes. (a) NNAL formation; (b) total α-carbon hydroxylation; (c) total N-oxidation. Data are presented as the means of duplicate incubations. N/D = Not Detectable.
carbon hydroxylation) (p<0.05) (figure 2.3(a)). In the presence of arachidonic acid, only keto acid and keto alcohol were detected and the levels of keto alcohol were significantly lower than those of keto acid (p<0.05) (figure 2.3(b)).

When comparing metabolite formation in the presence of the NADPH-generating system to metabolite formation in the presence of arachidonic acid, a 99% decrease in carbonyl reduction (figure 2.4(a)), 62% decrease in total α-carbon hydroxylation (figure 2.4(b)) and 88% decrease in total N-oxidation (figure 2.4(c)) was observed in the presence of arachidonic acid.

No significant differences in carbonyl reduction and α-carbon hydroxylation were observed in the presence of arachidonic acid compared to the absence of the NADPH-generating system (p>0.05) (figures 2.5(a) and (b)). Metabolites reflective of N-oxidation were not detected in the absence of NADPH but were present at a level of 0.000668 ± 0.000818% (mg protein)^-1 minute^-1 when arachidonic acid was added; this value was only slightly above the lower limit of detection for the metabolites.

2.3.3 Effects of Inhibitors on [5-3H]NNK Biotransformation in Human Lung Microsomes

The effects of chemical inhibitors on NNK biotransformation in individual patients are displayed in tables (tables A1.1, A1.2 and A1.3 in appendix 1) and graphs (figures 2.6, 2.7, 2.8 and 2.9).

In general, the addition of chemical inhibitors did not result in a substantial decrease in α-carbon hydroxylation, N-oxidation or carbonyl reduction of NNK.
Figure 2.3: [5-³H]NNK bioactivation into specific α-carbon hydroxylation end-point metabolites in human lung microsomes. (a) In the presence of the NADPH-generating system; (b) in the presence of arachidonic acid. Results presented as means ± S.D. from 4 patients.

*Significantly different from keto acid, repeated measures ANOVA.
≠Significantly different from keto acid, Student’s t test.
Figure 2.4: Metabolism of [5-3H]NNK in the presence of the NADPH-generating system and in the presence of arachidonic acid in human lung microsomes. (a) NNAL formation; (b) total α-carbon hydroxylation; (c) total N-oxidation. Results presented as means ± S.D. from 4 patients.

*Significantly different from + NADPH, Student’s t test.
Figure 2.5: Comparing [5-³H]NNK metabolism in the absence of the NADPH-generating system and in the presence of arachidonic acid in human lung microsomes. (a) NNAL formation; (b) total α-carbon hydroxylation; (c) total N-oxidation. Results presented as means ± S.D. from 4 patients. N/D = Not Detectable.
2.3.3.1 Individual Patient Results (in the Presence of NADPH)

In the presence of NADPH and indomethacin, inhibition of carbonyl reduction ranged from 76% to 98% (figures 2.7(a), 2.8(a) and 2.9(a)), inhibition of total $\alpha$-carbon hydroxylation ranged from 52% to 89% (figures 2.7(b), 2.8(b) and 2.9(b)) and inhibition of total $N$-oxidation ranged from 17% to 100% (figures 2.7(c), 2.8(c) and 2.9(c)). Indomethacin decreased total $N$-oxidation in patients 7M and 8M such that metabolite levels fell below the lower limit of detection (figures 2.7(c) and 2.8(c)). Data for patient 6M is unavailable due to problems with HPLC apparatus.

When microsomes were incubated with SC-560 or NS-398, the inhibition in metabolite formation was less than with indomethacin and, in some microsomes, a slight increase in metabolite formation was observed (figures 2.7, 2.8 and 2.9). The isoform-selective inhibitors caused a slight decrease in total $\alpha$-carbon hydroxylation and total $N$-oxidation in patient 7M (figures 2.7(b) and (c)). For patients 8M and 3J, the inhibitors caused an increase in total $\alpha$-carbon hydroxylation; the percentage increase was higher in patient 8M compared to patient 3J (figures 2.8(b) and 2.9(b)). Both SC-560 and NS-398 caused a decrease in total $N$-oxidation in patients 8M and 3J (figures 2.8(c) and 2.9(c)). Both isoform-selective inhibitors had a profound effect on carbonyl reduction in patient 8M (figure 2.8(a)); SC-560 decreased carbonyl reduction by 83% and NS-398 decreased carbonyl reduction by 63%. The isoform-selective inhibitors did not seem to greatly effect this pathway in any of the other patients.
Figure 2.6: Effects of 10 µM indomethacin, 100 nM SC-560 and 10 µM NS-398 on [5-3H]NNK biotransformation in lung microsomes of patient 6M in the presence of NADPH (white bars) and arachidonic acid (grey bars). (a) NNAL formation; (b) total α-carbon hydroxylation; (c) total N-oxidation. Results presented as means of duplicate reactions. N/D = Not Detectable, N/A = Not Available.
Figure 2.7: Effects of 10 µM indomethacin, 100 nM SC-560 and 10 µM NS-398 on [5-3H]NNK biotransformation in lung microsomes of patient 7M in the presence of NADPH (white bars) and arachidonic acid (grey bars). (a) NNAL formation; (b) total α-carbon hydroxylation; (c) total N-oxidation. Results presented as means of duplicate reactions. N/D = Not Detectable.
Figure 2.8: Effects of 10 μM indomethacin, 100 nM SC-560 and 10 μM NS-398 on [5-³H]NNK biotransformation in lung microsomes of patient 8M in the presence of NADPH (white bars) and arachidonic acid (grey bars). (a) NNAL formation; (b) total α-carbon hydroxylation; (c) total N-oxidation. Results presented as means of duplicate reactions. N/D = Not Detectable.
Figure 2.9: Effects of 10 µM indomethacin, 100 nM SC-560 and 10 µM NS-398 on [5-3H]NNK biotransformation in lung microsomes of patient 3J in the presence of NADPH (white bars) and arachidonic acid (grey bars). (a) NNAL formation; (b) total α-carbon hydroxylation; (c) total N-oxidation. Results presented as means of duplicate reactions. N/D = Not Detectable.
2.3.3.2 Individual Patient Results (in the Presence of Arachidonic Acid)

In the presence of arachidonic acid and indomethacin, the inhibition of α-carbon hydroxylation ranged from 33% to 67% (table A1.1), the inhibition of N-oxidation ranged from 80% to 100% (table A1.2) and the inhibition of carbonyl reduction ranged from 33% to 100% (table A1.3). The level of metabolites reflective of N-oxidation was below the lower limit of detection in the presence of arachidonic acid in patients 6M (figure 2.6), 7M (figure 2.7) and 3J (figure 2.9).

Both isoform-selective PHS inhibitors, SC-560 and NS-398, inhibited α-carbon hydroxylation by 21% in patient 6M (table A1.1 and figure 2.6(b)) and inhibited carbonyl reduction by 8 and 17%, respectively (table A1.3 and figure 2.6(a)). In patient 7M, the inhibitors did not cause a decrease in bioactivation (table A1.1 and figure 2.7(b)) and only produced a slight inhibition in carbonyl reduction (table A1.3 and figure 2.7(a)). For patients 8M and 3J, SC-560 caused a decrease in bioactivation such that metabolite levels fell below the lower limit of detection however, the addition of NS-398 appeared to result in an increase in metabolite formation in both patients (table A1.1 and figures 2.8(b) and 2.9(b)). Carbonyl reduction was only observed in patients 6M and 7M (table A1.3 and figures 2.6(a) and 2.7(a)); the isoform-selective inhibitors did not appear to have a profound effect on carbonyl reduction in these patients. Metabolites reflective of N-oxidation were only observed in patients 8M and 3J and the presence of SC-560 and NS-398 resulted in an inhibition in N-oxidation such that metabolite levels fell below the lower limit of detection (table A1.2 and figures 2.8(c) and 2.9(c)).
2.3.3.3 **Grouped Patient Results (in the presence of NADPH)**

Of the three inhibitors used, only one had a significant effect on NNK biotransformation. In the presence of 10 µM indomethacin, carbonyl reduction was inhibited by 92% (figure 2.10(a)), total α-carbon hydroxylation was inhibited by 71% (figure 2.10(b)) and total N-oxidation was inhibited by 93% (figure 2.10(c)). The addition of 100 nM SC-560 and 10 µM NS-398 did not significantly inhibit the formation of metabolites reflective of α-carbon hydroxylation, N-oxidation or carbonyl reduction (p>0.05) (figure 2.10).

2.3.3.4 **Grouped Patient Results (in the presence of Arachidonic Acid)**

The addition of 10 µM indomethacin did not significantly affect α-carbon hydroxylation, N-oxidation or carbonyl reduction (p>0.05) (figure 2.11). Similarly, addition of 100 nM SC-560 and 10 µM NS-398 did not significantly alter α-carbon hydroxylation or carbonyl reduction (p>0.05) (figure 2.11).

2.3.4 **Cyclooxygenase Activity of Purified Ovine PHS-1 and PHS-2**

The cyclooxygenase activity of purified PHS-1 and PHS-2 was calculated based on oxygen consumption over a 5-minute time period. The supplier reported the cyclooxygenase activity of both enzymes to be 100 U; the activity of PHS-1 was calculated to be 96.2 U and that of PHS-2 was calculated to be 98.1 U, verifying that the cyclooxygenase activity of both enzymes was functional. Figure 2.12 is a representative tracing depicting the decrease in oxygen levels in the presence of PHS-2. The tracings for PHS-1 were similar.
**Figure 2.10**: Effects of 10 µM indomethacin, 100 nM SC-560 and 10 µM NS-398 on [5-³H]NNK biotransformation in the presence of the NADPH-generating system in human lung microsomes. (a) NNAL formation; (b) total α-carbon hydroxylation; (c) total N-oxidation. Results presented as means ± S.D. from 3 patients.

*Significantly different from NADPH control, repeated measures ANOVA (Tukey-Kramer post-hoc test).
Figure 2.11: Effects of 10 µM indomethacin, 100 nM SC-560 and 10 µM NS-398 on [5-^3^H]NNK biotransformation in the presence of arachidonic acid in human lung microsomes. (a) NNAL formation; (b) total α-carbon hydroxylation; (c) total N-oxidation. Results presented as means ± S.D. from 4 patients. N/D = Non Detectable.
**Figure 2.12:** Cyclooxygenase activity of PHS. Representative oxygen consumption tracings depicting the decrease in oxygen levels as PHS-2 catalyzes the incorporation of oxygen into arachidonic acid.
2.3.5 Peroxidase Activity of Purified Ovine PHS-1 and PHS-2

Peroxidase activity of the purified enzymes was verified to be functional by assessing the oxidation of TMPD, which is coupled to the reduction of PGG$_2$. TMPD is an easily oxidizable compound that serves as a reducing co-substrate for PHS during the peroxidase reaction, producing a highly coloured product that absorbs at 611 nm. Reactions contained 100 U or 1000 U of purified PHS-1 or PHS-2 and were initiated with arachidonic acid. After a 15 minute incubation, the absorbance of oxidized TMPD was determined at 611 nm in a UV spectrophotometer. The peroxidase activity of the enzymes was calculated using an equation that linked the absorbance of oxidized TMPD with the extinction coefficient of TMPD. The activity of PHS-1 was determined to be 98.3 U and the activity of PHS-2 was determined to be 95.7 U. An increase in the units of enzyme by ten-fold led to an increase in the absorbance of oxidized TMPD by approximately ten-fold (figure 2.13).

2.3.6 Metabolism of [5-³H]NNK in Hamster Liver Microsomes

As a positive control for NNK biotransformation and optimum HPLC conditions for metabolite detection, hamster liver microsomes were incubated with [5-³H]NNK in the presence and absence of the NADPH-generating system. Substantial levels of metabolites reflective of the three biotransformation pathways were observed in these reactions, and all four metabolites reflective of NNK bioactivation were detected (figure 2.14(a)). Similar to results from human lung microsomes, NNAL was the major metabolite detected, at a level of 2.35%
Figure 2.13: Oxidation of TMPD in the presence of PHS-1 and PHS-2. Results are presented as means of duplicate reactions.
Figure 2.14: Metabolism of [5-\textsuperscript{3}H]NNK. (a) In hamster liver microsomes and (b) in the presence of purified PHS-1 and PHS-2. Results presented as the means of duplicate reactions. N/D = Non Detectable.
(mg protein)^{-1} \text{ minute}^{-1} \) in the presence of the NADPH-generating system and 3.66 (mg protein)^{-1} \text{ minute}^{-1} \) in the absence of NADPH. Total \( \alpha \)-carbon hydroxylation was much higher in the presence of NADPH than in its absence; in the presence of NADPH, 5.58\% total \( \alpha \)-carbon hydroxylation (mg protein)^{-1} \text{ minute}^{-1} \) was detected whereas, in the absence of NADPH, only 0.0515\% \( \alpha \)-carbon hydroxylation (mg protein)^{-1} \text{ minute}^{-1} \) was detected. Similarly, for \( N \)-oxidation, metabolite levels were much higher in the presence of NADPH than in the absence (0.274\% (mg protein)^{-1} \text{ minute}^{-1} \) versus 0.003\% (mg protein)^{-1} \text{ minute}^{-1} \) (figure 2.14(a))

2.3.7 Metabolism of [5-^{3}H]NNK By Purified Ovine PHS-1 and PHS-2

Metabolites reflective of \( \alpha \)-carbon hydroxylation and \( N \)-oxidation were not detected when [5-^{3}H]NNK was incubated with 1000 U of purified PHS-1 or PHS-2 (figure 2.14(b)). The only metabolite that was detected was NNAL; 0.0000505\% NNAL (unit of enzyme)^{-1} \text{ minute}^{-1} \) was detected in reactions containing PHS-2. Addition of arachidonic acid to the incubations decreased formation of NNAL by 46.4\%; in the presence of arachidonic acid, NNAL was detected at a percentage of 0.0000280 (unit of enzyme)^{-1} \text{ minute}^{-1} \). When the NADPH-generating system was replaced with arachidonic acid and hematin, two PHS cofactors, no NNK metabolism was detected.

2.4 Discussion

The tobacco-specific nitrosamine NNK is present in significant quantities in unburned tobacco and tobacco smoke (Hecht, 1998). It is a selective inducer of
lung adenocarcinoma in all rodent species tested and is thought to be responsible for the rise in the number of cases of human lung adenocarcinomas (Thun et al., 1997).

To date, the enzymes involved in the pulmonary biotransformation of NNK have not all been identified. Characterization of these enzymes may lead to the development of novel chemopreventive therapies involving the inhibition of NNK bioactivation and/or the enhancement of NNK detoxification. While it is known that CYP enzymes are actively involved in NNK biotransformation, it is also accepted that other enzymes could play a role in this process, and two such enzymes are PHS-1 and PHS-2 (Smith et al., 1995; Rioux and Castonguay, 1998b).

Both PHS-1 and PHS-2 have been shown to play a role in the biotransformation of pulmonary toxicants, such as B[a]P (Panthananickal and Marnett, 1981; Marnett et al., 1977; Marnett and Reed, 1979), aflatoxin B₁ (Battista and Marnett, 1985; Liu et al., 1990) and 4-aminobiphenyl (Wiese et al., 2001). In addition, NNK biotransformation by purified PHS-1 and PHS-2 has been reported previously (Rioux and Castonguay, 1998b). However, there were several problems with that study: the cofactors used in the reactions were more favourable with CYP-mediated biotransformation than with PHS-mediated biotransformation; the studies were performed in mouse lung microsomes and PHS-mediated NNK biotransformation in the human lung was not examined; and reported metabolite formation occurred at levels much higher than those previously detected by our laboratory and by other researchers. Therefore, it was the aim of the present study to verify the previous findings and to establish
whether or not PHS-1 and PHS-2 take part in NNK biotransformation in human lung.

Biotransformation of NNK in human lung microsomes from four patients (three males and one female) resulted in the formation of metabolites reflective of $\alpha$-carbon hydroxylation (keto acid, keto alcohol and diol), $N$-oxidation (NNK-$N$-oxide and NNAL-$N$-oxide) and carbonyl reduction (NNAL). The reactions were performed in the presence of an NADPH-generating system and in the presence of arachidonic acid.

Consistent with previous studies (Smith et al., 1995; Smith et al., 1999; Smith et al., 2003), NNAL was the major metabolite detected in all reactions with NADPH. Keto alcohol was the major $\alpha$-carbon hydroxylation metabolite formed in the presence of NADPH and in the presence of arachidonic acid. The higher levels of keto acid and keto alcohol were likely due to the presence of higher amounts of the precursor, NNK, compared to levels of the hydroxy acid and diol precursor, NNAL, since NNAL was produced from NNK. The overall balance between $\alpha$-carbon hydroxylation (bioactivation) and $N$-oxidation (detoxification) favoured bioactivation.

Substitution of the NADPH-generating system with arachidonic acid resulted in a significant decrease in metabolite levels reflective of all three biotransformation pathways. When NNK biotransformation in the absence of NADPH was compared to that in the presence of arachidonic acid, no significant differences in $\alpha$-carbon hydroxylation or carbonyl reduction were observed, suggesting that metabolite formation occurred due to residual NADPH and not as
a result of the addition of cofactors favourable with PHS-mediated biotransformation.

In the presence of the NADPH-generating system, indomethacin inhibited NNK biotransformation to various extents in all patients’ microsomes. Results from incubations in the presence of arachidonic acid showed a similar trend, although the magnitude of the effect was lower in the presence of arachidonic acid. The concentration of indomethacin used was consistent with what was previously reported for PHS inhibition (Smith and DeWitt, 1995; Smith et al., 2003). However it has been suggested that indomethacin, at this concentration, may be able to inhibit CYPs as well as PHS (Smith et al., 2003). Therefore, the indomethacin-mediated decrease in metabolite formation may have occurred as a result of inhibition of CYPs and/or PHS. For this reason, inhibitory studies were also performed in the presence of isoform-selective inhibitors.

SC-560 and NS-398 had limited effects on NNK biotransformation. SC-560 is highly selective for PHS-1 inhibition, with a median inhibitory concentration (IC$_{50}$) of 9 nM for human PHS-1 and 6.3 μM for human PHS-2. Similarly NS-398 is highly selective for PHS-2 inhibition, with an IC$_{50}$ of 1.9 μM for human PHS-2 compared to 75 μM for human PHS-1 (Hermenegildo et al., 2005). These chemicals have been widely used as selective inhibitors of PHS-1 and PHS-2 (Copeland et al., 1994; Mazieres et al., 2005; Sadovsky et al., 2000; Knorth et al., 2004).

In the presence of the NADPH-generating system, SC-560 caused a pronounced decrease in NNAL formation in one out of the four patients. A similar decrease was seen in the presence of NS-398. SC-560 decreased NNK
bioactivation and detoxification by less than 10% in the lung microsomes of patients 7M and 8M. Similarly, NS-398 caused a decrease in N-oxidation of less than 10% in patient 7M. Because metabolite levels were so low to begin with (total α-carbon hydroxylation accounted for much less than 1% the initial amount of NNK), these slight decreases in metabolite formation conceivably could be attributed to experimental variability. Both inhibitors increased NNK bioactivation in the lung microsomes of patients 8M and 3J. Although different inhibitors were used, this paradoxical increase in metabolite formation in the presence of inhibitors has been observed previously (Smith et al., 2003). The increase in metabolite formation may have occurred as a result of enzyme induction by tobacco smoke particulate matter that could have accumulated in the lungs of these patients.

In the presence of arachidonic acid, SC-560 inhibited NNK bioactivation in the microsomes from three of four patients, such that metabolite levels decreased by 21% (patient 6M) or fell below the lower limit of quantifiable detection (patients 8M and 3J). However, the effect was not statistically significant when results from the different patients were grouped. This could have been explained by PHS-1 contributing to NNK metabolism in some individuals. Alternatively, it is possible that SC-560 inhibited CYP-catalyzed NNK metabolism occurring due to residual NADPH in microsomes without added NADPH; apparently, no studies have yet examined the effects of SC-560 on CYP activity. Individual and grouped patient results with NS-398 suggest that PHS-2 does not play a role in NNK biotransformation.
Overall, results from microsomal incubations suggested that PHS-2 does not play a role in NNK biotransformation, while the potential contribution of PHS-1 remained equivocal. To address this ambiguity in microsomal results, it was important to determine if PHS-1 and PHS-2 actually have the ability to catalyze the biotransformation of NNK. PHS-1 and PHS-2 were obtained commercially and were purified from ram seminal vesicles and sheep placenta, two sources that are rich in PHS-1 and PHS-2, respectively (Duan and Zhang, 2006).

Initial experiments performed with PHS-1 and PHS-2 followed a methodology outlined by Rioux and Castonguay (1998b). The results show that neither PHS-1 nor PHS-2, both of which has been established to be catalytically active, catalyze NNK bioactivation and detoxification in the presence of the NADPH-generating system. The lone metabolite that could be detected was NNAL, which was formed only in the presence of PHS-2. These results are contradictory to those obtained by Rioux and Castonguay, who reported high levels of NNK metabolites reflective of all three biotransformation pathways in the presence of both PHS isoforms.

PHS-1 was reported by the supplier to be approximately 95% pure and PHS-2 was approximately 70% pure. The specific enzymes contaminating PHS-2 are unknown to the Cayman Chemical Company (personal communication). Hence, it is possible that one of the contaminating enzymes is a carbonyl reductase, and it is this enzyme that is responsible for the formation of NNAL in the presence of NADPH and PHS-2. The inability to detect NNAL formation in the presence of arachidonic acid and hematin further supports this theory. In order to function, carbonyl reductase requires the presence of NADPH (Wermuth,
1981; Matsuura et al., 1988) so, when the NADPH-generating system was replaced with arachidonic acid and hematin, carbonyl reductase lost the cofactor it required to catalyze the formation of NNAL.

In their study, Rioux and Castonguay (1998b) reported detection of α-carbon hydroxylation metabolites at levels as high as 5.5% for PHS-1 and 8.9% for PHS-2. These metabolite levels are 10,000 times greater than the lower limit of quantifiable detection and studies performed with hamster liver microsomes verified that the HPLC conditions were optimal for metabolite detection. Therefore, metabolite formation would have been easily detected if our levels were similar to what Rioux and Castonguay reported.

Previous work has demonstrated that compounds bioactivated by ovine PHS-1 and PHS-2 are also bioactivated by the human PHS isoforms (Liu et al., 1995; Wiese et al., 2001). In many cases, one isoform is the more active metabolizer than the other; for example, ovine PHS-2 is almost three times more active than ovine PHS-1 in the bioactivation of 2-aminofluorene (Liu et al., 1995). Similarly, human PHS-2 is approximately four times more active than human PHS-1 in 2-aminofluorene bioactivation (Liu et al., 1995). Another example is seen with the bioactivation of 4-aminobiphenyl; in this case, both ovine and human PHS-1 are more effective metabolizers than ovine and human PHS-2 (Wiese et al., 2001). It has also been observed that, in some instances, the human isoforms are more effective bioactivators and, in other cases, the ovine isoforms are more effective bioactivators. For example, human PHS-1 and PHS-2 are approximately three and five times more active than their ovine counterparts in the bioactivation of B[a]P-7,8-diol (Wiese et al., 2001).
Conversely, ovine PHS-1 and PHS-2 are much more active than human PHS-1 and PHS-2 in the bioactivation of benzidine (Wiese et al., 2001). Even though there are differences in the bioactivation activity by ovine and human PHS-1 and PHS-2, studies have demonstrated that both species’ enzymes can metabolize the same compound. This suggests that xenobiotic bioactivation by ovine PHS is a good indicator for xenobiotic bioactivation by human PHS and, therefore, the inability of ovine PHS-1 and PHS-2 to bioactivate NNK suggests an unlikely role for their human counterparts in this process.
Chapter 3

Investigation of the Biotransformation of
4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) by
Cytochrome P450 (CYP) 2F

3.1 Introduction

The CYP proteins are a superfamily of heme-containing enzymes capable of biotransforming endogenous and exogenous compounds into metabolites that can be active in vivo, can be further metabolized, or can be excreted from the body (Carr et al., 2003b). A number of human CYPs have been implicated in the pulmonary biotransformation of NNK (reviewed in section 1.4.2.2). However, there remain enzymes involved in this process that have yet to be identified. Another CYP subfamily that could be of particular importance in NNK metabolism is CYP2F.

The CYP2F subfamily is unusual for two reasons; first, CYP2F proteins are selectively expressed in the lung, with little or no expression in the liver or any other organs (Carr et al., 2003b). Second, the subfamily contains only a single functional enzyme in each species examined; 2F1 in humans, 2F2 in mice, 2F3 in goat and 2F4 in rats (Chen et al., 2002). The nucleic acid and amino acid sequence similarity between CYP2F homologues of different species is approximately 80 to 84% (Simmonds et al., 2004).

The ability of the CYP2F enzyme to bioactivate the lung toxicants 3-methylindole (3-MI), naphthalene and styrene has been demonstrated previously...
(Lanza et al., 1999; Nakajima et al., 1994). Of the three toxicants, 3-MI is particularly relevant to the research presented in this thesis as it can undergo selective and preferential dehydrogenation by CYPs 2F1 and 2F3, leading to formation of the highly reactive intermediate, 3-methyleneindolenine (3-MEI) (Yan et al., 2007) (figure 3.1). This intermediate can form covalent bonds with amino acids in the active sites of CYPs 2F1 and 2F3 and is a mechanism-based inactivator of these enzymes (Racha et al., 1998). Hence, 3-MI has been used as an inhibitor of CYPs 2F1 and 2F3 in our studies.

With regards to NNK, CYP2F1 expressed in human hepatoma G2 cells was demonstrated to biotransform the carcinogen (Smith et al., 1992), but low expression of CYP2F1 in those cells has been criticized because it makes quantification of NNK metabolism difficult (Dr. Garold S. Yost, personal communication). The objective of the present study was to establish the ability of CYP2F to biotransform NNK.

### 3.2 Materials and Methods

#### 3.2.1 Chemicals

Chemicals were obtained from suppliers as follows: nicotinamide adenine dinucleotide phosphate (NADPH, reduced form), 3-MI, 2-benzozolinone and chlorzoxazone from Sigma-Aldrich (St. Louis, MO). The sources for all other chemicals are listed in section 2.2.1.
Figure 3.1: Bioactivation of 3-MI via dehydrogenation to form the reactive metabolite 3-MEI. CYPs 2F1 and 2F3 specifically catalyze the dehydrogenation of 3-MI; the mechanism involves hydrogen abstraction at the methyl group followed by a one electron oxidation to form 3-MEI (Yan et al., 2007). The reactive intermediate is generated without the concurrent formation of the oxygenated metabolites, indole-3-carbinol (I3C) and 3-methyloxindole (3-MOI) (Lanza et al., 1999; Carr et al., 2003a).
3.2.2 Preparation of Recombinant CYP2F3

Recombinant CYP2F3 was provided by Dr. Garold S. Yost (Department of Pharmacology and Toxicology, University of Utah). The isolation, characterization and expression of CYP2F3 were performed at the University of Utah. The methodology is summarized in appendix 2 and outlined in figure A.2.1.

3.2.3 Procurement of Lung Tissue

Sections of peripheral lung tissue devoid of macroscopically visible tumours were obtained from patients during clinically indicated lobectomies at the Kingston General Hospital, as described in section 2.2.2. The tissue specimens were cut into 1.5 cm$^3$ pieces, wrapped in aluminium foil and stored at -80°C until required for microsome preparation.

3.2.4 Preparation of Human Lung Microsomes

Pieces of lung tissue were thawed, chopped and homogenized. Microsomes were prepared by differential centrifugation as described in section 2.2.3. Microsomal protein levels were determined according to the Lowry method using bovine serum albumin as the standard (Lowry et al., 1951).

3.2.5 Purification of [5-$^3$H]NNK

Prior to the start of experimentation, the purity of radiolabelled [5-$^3$H]NNK was determined. If the purity was less than 98%, [5-$^3$H]NNK was re-purified as described in section 2.2.4 before experiments began.
3.2.6 Purified Recombinant CYP2F3 Incubations with [5-\(^3\)H]NNK

Incubations contained 100 pmol CYP2F3 and cytochrome P450 reductase microsomes in a 1:3 ratio and 0.1 M potassium phosphate buffer (pH 7.4) in a total volume of 0.5 mL. Reactions were initiated with 1.0 mM NADPH and were allowed to incubate at 37°C for 20 minutes in a shaking water bath. Reactions were stopped by the addition of 300 µL each of 25% zinc sulphate and saturated aqueous barium hydroxide solution, centrifuged at 2,500g for 5 minutes, frozen in liquid N\(_2\) and stored at -80°C until HPLC analysis. Control incubations contained boiled enzymes (with NADPH added to initiate the reactions) or incubations minus NADPH. For inhibition, CYP2F3 was pre-incubated with 150 µM 3-MI for 20 minutes at 37°C in a shaking water bath. 3-MI was dissolved in DMSO which constituted 1% (v/v) of the total incubation volume. Control incubations contained boiled microsomes and 1% DMSO (v/v).

3.2.7 Microsomal Incubations with [5-\(^3\)H]NNK

Human lung microsomes (1.0 mg/mL) were incubated with 4.2 µM [5-\(^3\)H]NNK in a total reaction volume of 1.0 mL. The reaction mixture also contained 5.0 mM glucose-6-phosphate, 2.0 U glucose-6-phosphate dehydrogenase, 1.25 mM NADP, 1.0 mM EDTA, 3.0 mM MgCl\(_2\) and 0.1 M potassium phosphate buffer (pH 7.4) plus 1.15% KCl. Following a pre-incubation of 10 minutes, reactions were initiated by addition of microsomes. Incubations were carried out for 15 minutes at 37°C in a shaking water bath, and were terminated by addition of 300 µL each of 25% zinc sulphate and saturated...
aqueous barium hydroxide solution. Reactions were centrifuged at 2500g and
the supernatants were frozen in liquid N$_2$ and stored at -80°C until analysis.
When employed, 3-MI (100 μM) was pre-incubated with the microsomes for 30
minutes at 37°C in a shaking water bath. 3-MI was dissolved in DMSO which
constituted 0.5% (v/v) of the total incubation volume. Control incubations
contained boiled microsomes and 0.5% DMSO (v/v). All reactions were
performed in duplicate.

3.2.8 Measurement of NNK Metabolism

Metabolites were quantified by reverse-phase HPLC with radiometric
detection as described in section 2.2.7.

3.2.9 Data Analysis

Microsomal data were presented as means ± S.D. Data from
recombinant CYP2F3 experiments are presented as the means of duplicate
reactions. Statistically significant differences in grouped microsomal data
following inhibitor treatment were determined by repeated measures ANOVA
followed by the Tukey-Kramer post-hoc test, using Graphpad Prism 4. When
heterogeneity of variance was revealed via Cochran’s test, a positive integer
(k=1) was added to each relevant data point (since some points were equal to
zero) and a log or reciprocal transformation was performed on the results prior to
conducting ANOVA. If homogeneity of variance was not achieved with the
transformed data, the Friedman non parametric test was used (Zivin and Bartko,
1976). P<0.05 was considered statistically significant in all cases.
3.3 Results

3.3.1 Patient Demographics

NNK biotransformation was examined in the lung microsomes of the same patients described previously (table 2.1). Patient demographics are detailed in section 2.3.1.

3.3.2 [5-3H]NNK Biotransformation: CYP2F3

When recombinant goat CYP2F3 was incubated with [5-3H]NNK, keto acid, keto alcohol and NNK N-oxide were detected. NNAL, hydroxy acid and diol formation were not detected. Addition of 3-MI to the incubations caused a 61% decrease in α-carbon hydroxylation and a decrease in N-oxidation such that metabolite levels fell below the lower limit of detection (10% (µmol enzyme)⁻¹ minute⁻¹) (figure 3.2).

3.3.3 [5-3H]NNK Biotransformation: Individual Patient Results

NNK biotransformation in lung microsomes of three patients (two males and one female) resulted in formation of measurable amounts of keto acid, diol, keto alcohol, NNAL-N-oxide, NNK-N-oxide and NNAL. The only NNK end-point metabolite not detected was hydroxy acid. The overall balance between total α-carbon hydroxylation (bioactivation) and total N-oxidation (detoxification) generally favoured bioactivation.

Addition of 3-MI resulted in a decrease in total α-carbon hydroxylation, total N-oxidation and carbonyl reduction in lung microsomes from all patients examined (figures 3.3, 3.4 and 3.5). The largest decrease in α-carbon
Figure 3.2: Metabolism of [5-³H]NNK by goat recombinant CYP2F3 alone and in the presence of 150 µM 3-MI. Results presented as means of duplicate incubations. N/D = Not Detectable.
hydroxylation (68.5% relative to α-carbon hydroxylation no solvent control) was observed in patient 8M (figure 3.4(b)) and the largest decrease in N-oxidation (76.0% relative to N-oxidation no solvent control) was seen in patient 3J (figure 3.5(c)).

The decreases in α-carbon hydroxylation (compared to the α-carbon hydroxylation no solvent control) ranged from 19.6% in patient 7M to 52.3% in patient 3J to 68.5% in patient 8M (figures 3.2, 3.3 and 3.4 (b)). Compared to the DMSO control, the decrease in α-carbon hydroxylation ranged from 41.5% in patient 3J to 47.4% in patient 7M to 63.3% in patient 8M (figures 3.2, 3.3 and 3.4 (b)).

Decreases in N-oxidation (compared to the no solvent control) ranged from 22.7% in patient 7M to 70.3% in patient 8M to 76.0% in patient 3J. Compared to the DMSO control, the decreases in N-oxidation ranged from 10.5% in patient 7M to 61.9% in patient 8M to 72.7% in patient 3J (figures 3.3, 3.4 and 3.5(c)).

Large decreases in carbonyl reduction (i.e. 21.0%) were only observed in patient 7M. The decreases in carbonyl reduction (compared to the no solvent carbonyl reduction control) ranged from 3.1% in patient 3J to 8.3% in patient 8M to 21.5% in patient 7M. Compared to the DMSO control, the decreases in carbonyl reduction ranged from 3.4% in patient 3J to 5.0% in patient 7M. In patient 8M, there was an 8% increase in carbonyl reduction compared to the DMSO control.
Figure 3.3: Effects of 100 µM 3-MI on [5-^{3}H]NNK biotransformation (relative to no solvent and DMSO controls) in lung microsomes of patient 7M. (a) NNAL formation; (b) total α-carbon hydroxylation; (c) total N-oxidation. Results presented as means of duplicate reactions.
Figure 3.4: Effects of 100 µM 3-MI on [5-³H]NNK biotransformation (relative to no solvent and DMSO controls) in lung microsomes of patient 8M. (a) NNAL formation; (b) total α-carbon hydroxylation; (c) total N-oxidation. Results presented as means of duplicate reactions.
Figure 3.5: Effects of 100 μM 3-MI on [5-3H]NNK biotransformation (relative to no solvent and DMSO controls) in lung microsomes of patient 3J. (a) NNAL formation; (b) total α-carbon hydroxylation; (c) total N-oxidation. Results presented as means of duplicate reactions.
3.3.4 [5-³H]NNK Biotransformation: Grouped Patient Results

When metabolism from the three individuals was grouped, no significant decreases in carbonyl reduction were seen when inhibitory incubations were compared with the no solvent and DMSO controls (p>0.05) (figure 3.6(a)). Decreases in α-carbon hydroxylation fell slightly short of statistical significance when compared with the no solvent and DMSO controls (p = 0.0604) (figure 3.6(b)). Decreases in N-oxidation were observed to be statistically significantly only when compared to the no solvent control (p<0.05) (figure 3.6(c)).

3.4 Discussion

It is known that human CYPs play a major role in the pulmonary biotransformation of NNK. However, many of the enzymes involved in this process have yet to be characterized. One of the pulmonary CYP subfamilies that could be important in NNK metabolism is CYP2F.

The research performed in this thesis utilized the pneumotoxicant 3-MI as a mechanism-based inactivator of CYPs 2F1 and 2F3. CYP-mediated biotransformation of 3-MI occurs via two pathways; oxygenation, producing the stable metabolites I³C and 3-MOI, and dehydrogenation, producing the highly reactive intermediate 3-MEI (Carr et al., 2003a) (figure 3.1). It is suggested that this metabolite is able to form covalent bonds in the active sites of the enzymes that catalyze its formation (Kartha and Yost, manuscript in preparation). Many CYPs, including 1A1, 1A2, 1B1, 2A6, 2C19 and 2D6, catalyze the formation of I³C and 3-MOI in human pulmonary tissue (Carr et al., 2003a) however, the
Figure 3.6: Metabolism of $[5^{3}\text{H}]$NNK in the Presence of 100 μM 3-MI and 0.5 % DMSO (control) in Human Lung Microsomes. (a) NNAL formation; (b) total $\alpha$-carbon hydroxylation; (c) total N-oxidation. Results presented as means ± S.D. from 3 patients. N/D = Not Detectable.

*Significantly different from control, repeated measures ANOVA
formation of 3-MEI is restricted to only a few CYPs; 1A1, 1A2 and 2F1 (Lanza and Yost, 2001). The goat enzyme CYP2F3 is also active in the formation of 3-MEI (Lanza and Yost, 2001). A recent study demonstrated the ability of 3-MI to inactivate CYPs 2F1 and 2F3 in a concentration and time-dependent manner (Kartha and Yost, manuscript in preparation).

In the presence of goat recombinant CYP2F3, measurable levels of keto acid, keto alcohol and NNK-N-oxide were detected. Detectable NNAL formation was not observed, indicating that goat CYP2F3 does not catalyze carbonyl reduction. In addition, hydroxy acid, diol and NNAL-N-oxide (metabolites formed from NNAL; figure 1.1) were not detected; this finding was expected since NNAL was not formed in the reactions. The presence of NNK-N-oxide suggests that CYP2F3 contributes to NNK detoxification.

The addition of 3-MI to human lung microsomes resulted in a decrease in carbonyl reduction from 3.1% in patient 3J to 8.3% in patient 8M to 21.5% in patient 7M. The decrease in N-oxidation ranged from 22.7% in patient 7M to 70.3% in patient 8M to 76.0% in patient 3J and the inhibition of α-carbon hydroxylation from 19.6% in patient 7M to 52.3% in patient 3J to 68.5% in patient 8M. This suggests that CYP2F1 is quite active in NNK bioactivation in these patients. However, it is also possible that the decrease in metabolite formation is due to the inhibition of CYP2F1 as well as CYPs 1A1 and 1A2, since the formation of the dehydrogenated metabolite is known to be catalyzed by these enzymes also (Lanza and Yost, 2001). In addition, the large decreases in N-oxidation observed in patients 8M and 3J suggest that CYP2F1 may be active in NNK detoxification in these patients. 3-MI also had a slight effect on carbonyl
reduction in all three patients suggesting that either CYP2F1 (or CYPs 1A1 and 1A2) contributes to NNAL formation or that 3-MI is an inhibitor of carbonyl reductase, the enzyme considered primarily responsible for formation of NNAL (Hecht, 1998). In support of the former, there is recent evidence that other CYPs are capable of converting NNK to NNAL (Brown et al., 2007).

In conclusion, results from the present study suggest that the CYP2F subfamily is involved in NNK biotransformation. Although grouped microsomal results did not reveal a statistically significant inhibition in carbonyl reduction, inhibition of α-carbon hydroxylation by 3-MI occurred to a variable extent in lung microsomes from all three patients, and was close to statistical significance when data were grouped, suggesting that a significant effect might be observed with a higher N value. Inhibition by 3-MI is mediated by the reactive intermediate, 3-MEI; it has been suggested that 3-MEI forms covalent bonds within the active sites of the enzymes that catalyze its formation (Kartha and Yost, manuscript in preparation). Since formation of 3-MEI is also catalyzed by CYPs 1A1 and 1A2, it is possible that these enzymes were also inhibited by addition of 3-MI to microsomes. However, these enzymes preferentially form the oxygenation metabolites rather than the dehydrogenation metabolite (Lanza and Yost, 2001), so they are less prone to inhibition than is CYP2F1. Nevertheless, inactivation of CYPs 1A1 and 1A2 may partially account for the inhibition in metabolite formation.

Similar to the results from human lung microsomes, goat CYP2F3-catalyzed NNK biotransformation also resulted in the formation of metabolites.
The results suggest that the CYP2F enzyme subfamily is involved in NNK biotransformation and that the CYP2F1 contribution varies between individuals.
General Discussion and Future Directions

4.1 General Discussion

The studies described in this thesis were performed in order to identify enzymes that could take part in the pulmonary biotransformation of the carcinogenic tobacco-specific nitrosamine, NNK.

As with many other chemical carcinogens, NNK requires enzymatic bioactivation in order to exert tumorigenicity. Both NNK and its keto reduced form, NNAL, undergo bioactivation via α-carbon hydroxylation. This results in the formation of reactive metabolites which can methylate or pyridyloxobutylate DNA, initiating NNK-induced carcinogenesis. Detoxification of NNK and NNAL occurs via N-oxidation.

To date, only some enzymes involved in the pulmonary biotransformation of NNK have been identified. NNK is recognized to be a potent and important pulmonary carcinogen (Hecht, 1998); it is thought to be associated with the increase in the levels of adenocarcinoma seen in Canada and the United States (Hoffmann et al., 1996; Hoffmann and Hoffmann, 1997). To that extent, characterization of the enzymes involved in NNK biotransformation is important as it could lead to the development of novel chemopreventive therapies and a potential decrease in the number of lung cancer cases. Three potential contributors to NNK metabolism in humans are PHS-1, PHS-2 and CYP2F1.
PHS-1 and PHS-2 bioactivate several pulmonary carcinogens including B[a]P (Panthananickal and Marnett, 1981; Marnett et al., 1977; Marnett and Reed, 1979), aflatoxin B₁ (Battista and Marnett, 1985; Liu et al., 1990) and 4-aminobiphenyl (Wiese et al., 2001). With regards to NNK, a previous study reported the ability of purified PHS-1 and PHS-2 to biotransform NNK, although the study in question was flawed in a number of ways. Therefore, the role of PHS in NNK biotransformation has not yet been conclusively demonstrated, and it was an objective of the studies outlined in this thesis to demonstrate whether or not PHS-1 and PHS-2 play a role in NNK biotransformation.

Initial studies were performed with human lung microsomes in the presence of the NADPH-generating system, which is required for CYP-mediated biotransformation, and arachidonic acid, which is a cofactor required for PHS-mediated biotransformation. The results of these studies did not suggest a role for either PHS-1 or PHS-2 in NNK biotransformation, as metabolite formation was significantly lower in the presence of arachidonic acid and could be attributed to the presence of residual levels of NADPH in the microsomes.

Although the addition of indomethacin in the presence of the NADPH-generating system resulted in a pronounced inhibition of formation of α-carbon hydroxylation and N-oxidation metabolites, the addition of isoform-selective PHS inhibitors failed to significantly alter metabolite formation. A similar trend was seen when incubations were performed in the presence of arachidonic acid. Further studies using commercially-obtained purified ovine PHS-1 and PHS-2 also did not point to a role for PHS in NNK biotransformation. Previous work has shown that ovine and human PHS-1 and PHS-2 share a high degree of substrate
specificity (Liu et al, 1995; Wiese et al, 2001) and, given the inability of ovine PHS isoforms to bioactivate NNK, it is highly unlikely that human PHS isoforms are involved in this process.

The CYP2F subfamily has also been implicated in the bioactivation of lung toxicants, including 3-MI (Lanza et al., 1999), naphthalene (Lanza et al., 1999) and styrene (Nakajima et al., 1994). The ability of human CYP2F1 to biotransform NNK has not been conclusively demonstrated since the lone study to investigate this hypothesis was problematic (Smith et al., 1992). The second objective of this thesis was to determine whether or not a CYP2F subfamily member could biotransform NNK.

Initial studies performed with human lung microsomes in the presence of the CYP2F inactivator, 3-MI, did not reveal any significant decreases in NNK bioactivation and carbonyl reduction. However, when metabolite formation was examined in microsomes of individual subjects, decreases in NNK biotransformation were observed, and the inhibition was more pronounced in some individuals than others. In addition to inhibiting CYP2F1, 3-MI is known to be an inactivator of CYPs 1A1 and 1A2 and so the decrease in biotransformation may also be attributed to the inhibition of those enzymes. Nevertheless, the results suggest that CYP2F1 is involved in pulmonary NNK biotransformation and the role that it plays is more prominent in some individuals than others.

The incubation of goat recombinant CYP2F3 with NNK resulted in the formation of keto acid, keto alcohol and NNK-\textit{N}-oxide. This confirmed that CYP2F does play a role in the biotransformation of NNK, and also suggested the involvement of CYP2F in NNK detoxification. Both goat CYP2F3 and human
CYP2F1 have been shown to bioactivate various substrates (Wang et al., 1998; Lanza et al., 1999), which suggests that the catalytic activity of both enzymes are similar. Therefore, it is highly likely that NNK is bioactivated and detoxified by CYP2F1.

Overall, these studies demonstrate a role for CYP2F in pulmonary NNK metabolism, but strongly argue against the involvement of PHS enzymes.

4.2 Future Directions

While human CYP2F1 and goat CYP2F3 share a high degree of nucleic acid and amino acid sequence homology, it would be beneficial to examine NNK biotransformation by purified human CYP2F1. This is especially important since results from human lung microsomes were slightly ambiguous as 3-MI is not completely selective for CYP2F1 inhibition.

Recombinant vaccinia viruses expressing CYP2F1 have previously been used to examine NNK biotransformation (Thornton-Manning et al., 1996; Smith et al., 1992); however, the efficiency of CYP2F1 expression was poor and it was therefore difficult to assess how active the enzyme was in metabolizing NNK (Dr. Garold S. Yost, personal communication).

In addition, studies correlating microsomal CYP2F1 levels with levels of NNK metabolites could be carried out in order to determine how vital this enzyme is to NNK biotransformation. NNK metabolism could be correlated with CYP2F1 levels in human lung microsomes with the latter being determined by mass spectrometry. Unique amino acid sequences for CYP2F1 could be used to detect the enzyme and quantification could be done by mass spectrometry; this
novel approach is extremely sensitive and specific (Anderson and Hunter, 2006) which makes it highly useful for CYP quantification in human lung microsomes.


Appendix 1

Effects of PHS Inhibitors on NNK Biotransformation

The following tables detail the effects of the general PHS inhibitor indomethacin and the isoform selective PHS inhibitors SC-560 and NS-398 on α-carbon hydroxylation (table A1.1), N-oxidation (table A1.2) and carbonyl reduction (table A1.3) by. The graphs corresponding to the values in the tables can be seen in figures 2.6, 2.7, 2.8 and 2.9.
Table A1.1  Microsomal [5-3H]NNK total α-carbon hydroxylation in the absence or presence of inhibitors

<table>
<thead>
<tr>
<th>Microsomal Incubations</th>
<th>Microsomal Code(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6M</td>
</tr>
<tr>
<td>- NADPH</td>
<td>4.00</td>
</tr>
</tbody>
</table>

\(\text{(+)}\) NADPH:

<table>
<thead>
<tr>
<th></th>
<th>6M</th>
<th>7M</th>
<th>8M</th>
<th>3J</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADPH control</td>
<td>14.3(^b)</td>
<td>15.3</td>
<td>3.00</td>
<td>14.0</td>
</tr>
<tr>
<td>+ 10 μM Indomethacin (PHS)(^c)</td>
<td>N/A(^d)</td>
<td>7.33 (-52)(^e)</td>
<td>0.333 (-89)</td>
<td>1.67 (-80)</td>
</tr>
<tr>
<td>+ 100 nM SC-560 (PHS-1)</td>
<td>N/A</td>
<td>14.3 (-6.5)</td>
<td>3.67 (+22)</td>
<td>14.7 (+5)</td>
</tr>
<tr>
<td>+ 10 μM NS-398 (PHS-2)</td>
<td>N/A</td>
<td>13.3 (-13)</td>
<td>4.17 (+39)</td>
<td>15.0 (+7)</td>
</tr>
</tbody>
</table>

\(\text{(+)}\) 300 mM Arachidonic acid:

<table>
<thead>
<tr>
<th></th>
<th>6M</th>
<th>7M</th>
<th>8M</th>
<th>3J</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arachidonic Acid control</td>
<td>4.67</td>
<td>6.00</td>
<td>2.50</td>
<td>4.33</td>
</tr>
<tr>
<td>+ 10 μM Indomethacin (PHS)</td>
<td>1.67 (-64)</td>
<td>4.00 (-33)</td>
<td>0.833 (-67)</td>
<td>2.33 (-46)</td>
</tr>
<tr>
<td>+ 100 nM SC-560 (PHS-1)</td>
<td>3.67 (-21)</td>
<td>6.00 (0)</td>
<td>N/D</td>
<td>N/D</td>
</tr>
<tr>
<td>+ 10 μM NS-398 (PHS-2)</td>
<td>3.67 (-21)</td>
<td>6.00 (0)</td>
<td>3.00 (+20)</td>
<td>5.00 (+15)</td>
</tr>
</tbody>
</table>

Abbreviations: \(\text{+}\) = incubations in the presence of; \(-\) = incubations in the absence of; N/D = not detected, N/A = not available
\(^a\)Microsomes are assigned codes for patient confidentiality
\(^b\)Data are presented as the sum of four α-hydroxylation end-point metabolites (% (mg protein)\(^{-1}\) minute\(^{-1}\) x 10\(^3\))
\(^c\)Target biotransformation enzyme of chemical inhibitor
\(^d\)Not available due to problems with chromatography apparatus
\(^e\)Numbers in brackets represent % change in total NNK bioactivation
Table A1.2  Microsomal [5-<sup>3</sup>H]NNK total N-oxidation in the absence or presence of inhibitors

<table>
<thead>
<tr>
<th>Microsomal Incubations</th>
<th>Microsomal Code&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6M</td>
</tr>
<tr>
<td>- NADPH</td>
<td>N/D</td>
</tr>
<tr>
<td>(+) NADPH:</td>
<td></td>
</tr>
<tr>
<td>NADPH control</td>
<td>6.67&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>+ 10 µM Indomethacin (PHS)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>N/A&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>+ 100 nM SC-560 (PHS-1)</td>
<td>N/A</td>
</tr>
<tr>
<td>+ 10 µM NS-398 (PHS-2)</td>
<td>N/A</td>
</tr>
<tr>
<td>(+) 300 mM Arachidonic acid:</td>
<td></td>
</tr>
<tr>
<td>Arachidonic Acid control</td>
<td>N/D</td>
</tr>
<tr>
<td>+ 10 µM Indomethacin (PHS)</td>
<td>N/D</td>
</tr>
<tr>
<td>+ 100 nM SC-560 (PHS-1)</td>
<td>N/D</td>
</tr>
<tr>
<td>+ 10 µM NS-398 (PHS-2)</td>
<td>N/D</td>
</tr>
</tbody>
</table>

Abbreviations: + = incubations in the presence of; - = incubations in the absence of; N/D = not detected, N/A = not available

<sup>a</sup>Microsomes are assigned codes for patient confidentiality

<sup>b</sup>Data are presented as the sum of two N-oxidation metabolites (% (mg protein)<sup>-1</sup> minute<sup>-1</sup> x 10<sup>4</sup>)

<sup>c</sup>Target biotransformation enzyme of chemical inhibitor

<sup>d</sup>Not available due to problems with chromatography apparatus

<sup>e</sup>Numbers in brackets represent % change in total NNK detoxification
<table>
<thead>
<tr>
<th>Microsomal Incubations</th>
<th>Microsomal Code&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6M</td>
</tr>
<tr>
<td>- NADPH</td>
<td>0.233</td>
</tr>
<tr>
<td>(+) NADPH:</td>
<td></td>
</tr>
<tr>
<td>NADPH control</td>
<td>31.3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>+ 10 μM Indomethacin (PHS)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>N/A&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>+ 100 nM SC-560 (PHS-1)</td>
<td>N/A</td>
</tr>
<tr>
<td>+ 10 μM NS-398 (PHS-2)</td>
<td>N/A</td>
</tr>
<tr>
<td>(+) 300 mM Arachidonic acid:</td>
<td></td>
</tr>
<tr>
<td>Arachidonic acid control</td>
<td>0.400</td>
</tr>
<tr>
<td>+ 10 μM Indomethacin (PHS)</td>
<td>0.267 (-33)</td>
</tr>
<tr>
<td>+ 100 nM SC-560 (PHS-1)</td>
<td>0.367 (-8.0)</td>
</tr>
<tr>
<td>+ 10 μM NS-398 (PHS-2)</td>
<td>0.333 (-17)</td>
</tr>
</tbody>
</table>

Abbreviations: + = incubations in the presence of; - = incubations in the absence of; N/D = not detected, N/A = not available

<sup>a</sup> Microsomes are assigned codes for patient confidentiality
<sup>b</sup> Data are presented as (% (mg protein)<sup>-1</sup> minute<sup>-1</sup> x 10<sup>2</sup>)
<sup>c</sup> Target biotransformation enzyme of chemical inhibitor
<sup>d</sup> Not available due to problems with chromatography apparatus
<sup>e</sup> Numbers in brackets represent % change in NNAL formation
**Appendix 2**

**NNK Biotransformation by CYP2F**

**A2.1 Preparation of Recombinant CYP2F3**

Recombinant CYP2F3 was provided to the laboratory by Dr. Garold S. Yost (Department of Pharmacology and Toxicology, University of Utah). The methodology for the isolation, characterization and expression of CYP2F3 (performed at the University of Utah) is detailed in the following paragraphs and outlined in figure A2.1. Recombinant CYP2F3 was used in the experiments described in section 3.2.7.

**A2.1.1 Isolation and Characterization of CYP2F3**

Goat lung CYP2F3 cDNA was cloned into the EcoRI restriction site of the pBluescript SK phagemid present in the Lambda ZAP II expression vector. Vectors were screened for CYP2F3 cDNA using a human CYP2F1 cDNA probe. Phagemids containing CYP2F3 cDNA were removed from the expression vector and transformed into bacteria. Bacterial colonies containing plasmid DNA and the cloned cDNA were selected from LB/ampicillin plates and plasmid DNA was extracted from the bacteria. Plasmid DNA underwent restriction digestion and southern blot analysis (using human CYP2F1 cDNA as a probe) was performed on the digested fragments. The longest cDNA fragments, containing both the start and stop codons, were selected and sequenced. These fragments were also used to synthesize a mutated cDNA fragment.
A2.1.2 Expression of CYP2F3 in *E. coli*

Mutant CYP2F3 cDNA was constructed with the addition of an *NdeI* restriction site upstream of the start codon and a *HindIII* restriction site downstream of the stop codon. Polymerase chain reactions were then performed using primers for this construct and the products were digested sequentially using *NdeI* and *HindIII* for insertion into the pCW expression vector. The CheW gene of the vector was excised by digesting the plasmid with *NdeI* and *HindIII* and the construct was introduced into the *E. coli* strain of DH5α cells. The transformed *E. coli* cells were selected on LB/ampicillin plates and cellular membrane fractions were prepared from the cell suspensions.

A2.1.3 Characterization of CYP2F3 Expression

The expression of CYP2F3 was calculated via carbon monoxide-dependent difference spectra, using an extinction coefficient of 91 cm⁻¹ mM⁻¹ for the difference of absorbance between 450 and 490 nm. Protein concentrations were estimated using the BCA protein assay reagents with bovine serum albumin as the standard.
Phagemid cloned into Lambda ZAP II expression vector

Phagemids containing CYP2F3 cDNA removed from expression vector and transformed into bacteria

Bacteria grown on LB/ampicillin plates. Bacterial colonies containing CYP2F3 cDNA selected and cDNA extracted

Start codon

ATG

Stop codon

TGA

NdeI restriction site

HindIII restriction site

Mutant CYP2F3 constructed

PCR performed using following primers (NdeI and HindIII sequences underlined):

5′-GCA ACT GCC TGC CAT ATG GCT CTG TTA TTA GCA GTT TTT TTG CTC CTG ATC-3′

5′-TCA CAG ACG AAG CTT GAA GGG-3′

PCR products digested with NdeI and HindIII and inserted into pCW expression vector. CheW gene (present after the NdeI cloning site) excised following digestion with NdeI and HindIII. CYP2F3 cDNA subcloned into E. coli strain DH5α cells. Transformed E. coli colonies selected on LB/ampicillin plates and cellular membrane fractions prepared from cell suspensions.

**Figure A2.1:** Isolation, characterization and expression of CYP2F3 (Wang et al., 1998).
A2.2 Effects of 3-MI on [5-\textsuperscript{3}H]NNK Biotransformation

The following table details the effects of 3-MI and DMSO metabolites representative of \(\alpha\)-carbon hydroxylation (hydroxy acid, keto acid, diol and keto alcohol), \(N\)-oxidation (NNAL-\(N\)-oxide and NNK-\(N\)-oxide) and carbonyl reduction (NNAL).
<table>
<thead>
<tr>
<th>Treatment Conditions per Microsomal Code(^a)</th>
<th>Hydroxy Acid</th>
<th>Keto Acid</th>
<th>NNAL-N-Oxide</th>
<th>Diol</th>
<th>NNK-N-Oxide</th>
<th>Keto Alcohol</th>
<th>NNAL</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>7M</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No solvent control</td>
<td>N/D</td>
<td>15.0(^b)</td>
<td>7.33</td>
<td>4.33</td>
<td>N/D</td>
<td>4.67</td>
<td>828</td>
</tr>
<tr>
<td>+ 0.5% DMSO</td>
<td>N/D</td>
<td>20.0</td>
<td>6.33</td>
<td>2.67</td>
<td>N/D</td>
<td>13.7</td>
<td>684</td>
</tr>
<tr>
<td>+ 100 μM 3-MI (2F1)(^c)</td>
<td>N/D</td>
<td>9.33</td>
<td>5.33</td>
<td>1.67</td>
<td>N/D</td>
<td>8.33</td>
<td>649</td>
</tr>
<tr>
<td><strong>8M</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No solvent control</td>
<td>N/D</td>
<td>15.3</td>
<td>9.00</td>
<td>6.33</td>
<td>N/D</td>
<td>1.67</td>
<td>591</td>
</tr>
<tr>
<td>+ 0.5% DMSO</td>
<td>N/D</td>
<td>18.7</td>
<td>7.00</td>
<td>N/D</td>
<td>N/D</td>
<td>1.33</td>
<td>335</td>
</tr>
<tr>
<td>+ 100 μM 3-MI (2F1)</td>
<td>N/D</td>
<td>6.33</td>
<td>1.33</td>
<td>2.33</td>
<td>N/D</td>
<td>N/D</td>
<td>542</td>
</tr>
<tr>
<td><strong>3J</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No solvent control</td>
<td>N/D</td>
<td>14.0</td>
<td>8.33</td>
<td>3.00</td>
<td>N/D</td>
<td>9.17</td>
<td>615</td>
</tr>
<tr>
<td>+ 0.5% DMSO</td>
<td>N/D</td>
<td>14.0</td>
<td>7.33</td>
<td>4.33</td>
<td>N/D</td>
<td>3.00</td>
<td>517</td>
</tr>
<tr>
<td>+ 100 μM 3-MI (2F1)</td>
<td>N/D</td>
<td>8.00</td>
<td>2.00</td>
<td>2.67</td>
<td>N/D</td>
<td>1.83</td>
<td>593</td>
</tr>
</tbody>
</table>

Abbreviations: + = incubations in the presence of; - = incubations in the absence of; N/D = not detected.

\(^a\)Microsomes are assigned codes for patient confidentiality

\(^b\)Data are presented as (% (mg protein\(^{-1}\) minute\(^{-1}\) x 10\(^3\))

\(^c\)Target biotransformation enzyme of chemical inhibitor