DNA OXIDATION AND BASE EXCISION REPAIR IN LUNG AND LIVER OF 4-(METHYLNITROSAMINO)-1-(3-PYRIDYL)-1-BUTANONE TREATED MICE

by

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A thesis submitted to the Department of Pharmacology and Toxicology
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Abstract

4-(Methylnitrosamo)ino)-1-(3-pyridyl)-1-butanone (NNK) is a potent pulmonary carcinogen found in unburned tobacco and tobacco smoke. To exert its carcinogenic effect, NNK is metabolically activated to reactive intermediates that can damage DNA by alkylation or pyridyloxobutylation. NNK also has the ability to induce DNA oxidation and alter DNA repair activities that can result in deficient repair and potentially exacerbate carcinogenesis. Base excision repair (BER) is a ubiquitous DNA repair system that mainly repairs oxidative DNA damage. The goal of this study was to determine the effect of NNK on DNA oxidation status and BER activity in A/J mouse lung and liver. Female mice were treated with 10 μmol of NNK i.p. and lung and liver were isolated 1, 2 and 24 hours post administration. DNA was isolated from lung and liver, and the formation of 8-hydroxydeoxyguanosine (8-OHdG, a biomarker of DNA oxidation) was assessed by high-performance liquid chromatography with electrochemical detection. At 1, 2 and 24 hours in both murine lung and liver, there was no statistically significant difference in 8-OHdG levels (n = 4, P > 0.05) between control and NNK-treated mice. To assess BER, cell-free whole tissue nuclear protein extracts from liver and lung were prepared and incubated with a plasmid substrate containing oxidative DNA damage. In vivo treatment with NNK did not alter BER activity in lung or liver compared to control mice (n=3 or 4, P > 0.05). These experiments indicate that acute treatment with a tumourigenic dose of NNK does not significantly stimulate oxidative DNA damage or significantly alter BER activity in murine lung and liver.

Keywords: 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, oxidative DNA damage, base excision repair
Co-Authorship

This research was conducted by the candidate, Neeraj Gupta, under the supervision of Dr. Thomas E. Massey.
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<th>Description</th>
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<tbody>
<tr>
<td>A</td>
<td>adenine</td>
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<tr>
<td>AC</td>
<td>adenocarcinoma</td>
</tr>
<tr>
<td><em>ad libitum</em></td>
<td>free feeding</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>amol</td>
<td>atto (10^{-18}) moles</td>
</tr>
<tr>
<td>AP</td>
<td>apurinic/apyrimidinic site</td>
</tr>
<tr>
<td>BER</td>
<td>base excision repair</td>
</tr>
<tr>
<td>bp</td>
<td>basepair</td>
</tr>
<tr>
<td>BP</td>
<td>benzo[(\alpha)]pyrene</td>
</tr>
<tr>
<td>C</td>
<td>cytosine</td>
</tr>
<tr>
<td>°C</td>
<td>degree(s) Celsius</td>
</tr>
<tr>
<td>Ci</td>
<td>curie(s)</td>
</tr>
<tr>
<td>cm</td>
<td>centimetre(s)</td>
</tr>
<tr>
<td>CYP</td>
<td>cytochrome P450</td>
</tr>
<tr>
<td>dATP</td>
<td>deoxyadenosine triphosphate</td>
</tr>
<tr>
<td>dCTP</td>
<td>deoxycytidine triphosphate</td>
</tr>
<tr>
<td>DFO</td>
<td>deferoxamine mesylate</td>
</tr>
<tr>
<td>2’-dG</td>
<td>2’-deoxyguanosine</td>
</tr>
<tr>
<td>dGTP</td>
<td>deoxyguanosine triphosphate</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>5’dRP</td>
<td>5’-deoxyribose-5-phosphate</td>
</tr>
<tr>
<td>dTTP</td>
<td>deoxythymidine triphosphate</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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</table>
e.g. exempli gratia (for example)
EtBr ethidium bromide
Fe iron
Fen1 flap endonuclease I
g gram(s)
g relative centrifugal force
G guanine
GC-MS gas chromatography-mass spectrometry
HEPES N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid
H$_2$O$_2$ hydrogen peroxide
HPB 4-hydroxy-1-(3-pyridyl)-1-butanone
HPLC-ECD high-performance liquid chromatography with electrochemical detection
hr hour(s)
i.e. id est (that is)
_in vitro_ outside the living body
_in vivo_ inside the living body
i.p. intraperitoneal
iso-NNAC 4-(methylnitrosamino)-4-(3-pyridyl)butyric acid
iso-NNAL 4-(methylnitrosamino)-4-(3-pyridyl)-1-butanol
kb kilobasepair
kg kilogram(s)
K-ras Kirsten-ras oncogene
Lig III DNA ligase III
μA microampere(s)
μCi microcurie(s)
μg microgram(s)
μL microlitre(s)
μM micromolar
μmol micromole(s)
M molar
min minute(s)
mg milligram(s)
7-mG 7-methylguanine
MGMT O^6^-methylguanine-DNA methyltransferase
mL millilitre(s)
mm millimetre(s)
mM millimolar
mRNA messenger ribonucleic acid
MUTYH adenine-DNA glycosylase
mV millivolt(s)
nA nanoampere(s)
NAB N\(^{i}\)-nitrosoanabasine
NAT N\(^{i}\)-nitrosoanatabine
NER nucleotide excision repair
ng nanogram(s)
nm nanometre(s)
NMWL nominal molecular weight limit
NNA 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanal
NNAL x
<table>
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<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>NNK</td>
<td>4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone</td>
</tr>
<tr>
<td>NNN</td>
<td>N'-nitrosornonnicotine</td>
</tr>
<tr>
<td>NO₂⁻</td>
<td>nitrite</td>
</tr>
<tr>
<td>NO₃⁻</td>
<td>nitrate</td>
</tr>
<tr>
<td>NSCLC</td>
<td>non-small cell lung cancer</td>
</tr>
<tr>
<td>^1O₂</td>
<td>singlet oxygen</td>
</tr>
<tr>
<td>O₂⁻</td>
<td>superoxide radical anion</td>
</tr>
<tr>
<td>OGG1</td>
<td>8-oxoguanine glycosylase 1</td>
</tr>
<tr>
<td>•OH</td>
<td>hydroxyl radicals</td>
</tr>
<tr>
<td>8-OHdG</td>
<td>8-hydroxydeoxyguanosine</td>
</tr>
<tr>
<td>O⁶-mG</td>
<td>O⁶-methylguanine</td>
</tr>
<tr>
<td>O¹-mT</td>
<td>O¹-methylthymine</td>
</tr>
<tr>
<td>OPB</td>
<td>4-oxo-4-(3-pyridyl)butanal</td>
</tr>
<tr>
<td>OPBA</td>
<td>4-oxo-4-(3-pyridyl)butyric acid</td>
</tr>
<tr>
<td>^32P</td>
<td>phosphorous-32</td>
</tr>
<tr>
<td>P &lt;</td>
<td>probability less than</td>
</tr>
<tr>
<td>P &gt;</td>
<td>probability greater than</td>
</tr>
<tr>
<td>p16</td>
<td>p16&lt;sub&gt;ink4a&lt;/sub&gt; tumour suppressor gene</td>
</tr>
<tr>
<td>PCNA</td>
<td>proliferating cell nuclear antigen</td>
</tr>
<tr>
<td>POB</td>
<td>pyridyloxobutyl</td>
</tr>
<tr>
<td>Polβ</td>
<td>DNA polymerase beta</td>
</tr>
<tr>
<td>RF-C</td>
<td>replication factor C</td>
</tr>
<tr>
<td>RGS</td>
<td>regulator of G-protein signaling</td>
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<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
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<td>ROS</td>
<td>reactive oxygen species</td>
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xi
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<tr>
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<tr>
<td>SCC</td>
<td>squamous cell cancer</td>
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<tr>
<td>SCLC</td>
<td>small cell lung cancer</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>T</td>
<td>thymine</td>
</tr>
<tr>
<td>TCDD</td>
<td>2,3,7,8-tetrachlorodibenzo-p-dioxin</td>
</tr>
<tr>
<td>TSNA</td>
<td>tobacco specific nitrosamines</td>
</tr>
<tr>
<td>U</td>
<td>enzymatic unit(s)</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>v/v</td>
<td>volume per volume</td>
</tr>
<tr>
<td>v/v/v</td>
<td>volume per volume per volume</td>
</tr>
<tr>
<td>W</td>
<td>watt(s)</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>x</td>
<td>times</td>
</tr>
<tr>
<td>XRCC1</td>
<td>X-ray repair cross-complementing protein</td>
</tr>
<tr>
<td>2xYT</td>
<td>yeast extract tryptone</td>
</tr>
<tr>
<td>~</td>
<td>approximately</td>
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<tr>
<td>&gt;</td>
<td>greater than</td>
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<td>&lt;</td>
<td>less than</td>
</tr>
<tr>
<td>±</td>
<td>plus or minus</td>
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Chapter 1
GENERAL INTRODUCTION

1.1 Statement of research problem
Lung cancer is currently the leading cause of cancer death worldwide. The widespread use of cigarettes and tobacco products was responsible for the rise in lung cancer incidence during the 20th century. A potent pulmonary carcinogen that most likely plays a major role in tobacco-induced lung cancer is 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). NNK is found in cigarettes and tobacco smoke and a substantial amount of experimental data has shown NNK to induce lung tumours in a variety of laboratory animals. Since cigarettes are widely available and consumed by so many, understanding the mechanism of action of major tobacco-specific carcinogens like NNK, is critical for developing novel preventative or treatment therapies for tobacco-induced lung cancer. The current goal of the research is to investigate the mechanism of action of NNK and in particular; 1) to assess the extent of DNA oxidation in murine lung and liver after in vivo treatment with NNK; 2) to determine if in vivo treatment with NNK can alter the activity of base excision repair (BER), a major DNA repair system.

1.2 Multi-stage process of carcinogenesis
Cancer can be defined as the loss of regulatory mechanisms that manage homeostasis and normal growth of cells. The transformation of a healthy cell into a cancerous one is dependent on the accumulation of a number of genetic and epigenetic changes. Abnormalities in the genome and epigenome (changes in gene expression caused by mechanisms that do not involve the DNA sequence) can alter the way a cell functions in its microenvironment and can result in cancer. These changes in cell function, dubbed the hallmarks of cancer, include upregulation of growth signals and receptors, insensitivity to growth inhibition, evasion of apoptosis, limitless replication
potential, sustained angiogenesis, tissue invasion and metastasis (Hanahan and Weinberg, 2000). The development of cancer typically follows a multistage process and can be described by a model involving three stages; initiation, promotion and progression.

1.2.1 Tumour initiation

The initiation stage of carcinogenesis involves the transformation of a healthy cell into a preneoplastic cell. During initiation, the molecular structure of DNA is irreversibly altered by an initiating agent (either chemical, physical or biological in nature) in a dose dependent manner (Pitot et al., 1981). The resulting products are initiated cells that are difficult to detect due to similar histology and phenotype to normal cells. Initiating agents and their metabolites introduce non-lethal damage to the genome and/or epigenome by covalently binding to DNA, inducing strand breaks, distorting the helix structure or modifying components such as nitrogenous bases or sugar-phosphate backbone (Pitot et al., 1981). It is well established that the DNA binding capacity of a carcinogen is correlated to its carcinogenic potency. Initiated cells require one round of DNA replication for the damage to be permanent (Bertram, 2000). An initiated cell will continue to exist and replicate unless the DNA damage is repaired or the cell is eliminated.

Abnormal alterations in DNA can be attributed to endogenous factors or exogenous agents such as UV light, ionizing radiation, and chemical carcinogens. Endogenous damage to DNA can arise from reactive molecular byproducts from normal cellular processes. Metabolism of oxygen during aerobic respiration is a major source of reactive oxygen species that can oxidize DNA and cause genetic mutations (Klaunig and Kamendulis, 2004). DNA also undergoes frequent spontaneous damage due to the inherent instability of the DNA helix structure. For example, in a given cell, depurination occurs $10^4$ times a day and is caused by the breakage of the $N$-glycosidic bond connecting nitrogenous base to deoxyribose sugar (Bertram, 2000). Apurinic
sites in DNA can result in random base insertion, potentially giving rise to mutations during replication or transcription.

Chemicals are of major importance in causing damage to DNA during the initiation stage of carcinogenesis. It is estimated that over 80% of all cancer deaths in industrialized nations are attributed to exogenous chemical factors found in diet, tobacco, alcohol, and occupational hazards (Luch, 2005). Most chemical carcinogens are not themselves directly carcinogenic. A few carcinogens like ethylene oxide, bis(chloromethyl) ether and nitrogen mustard gas are considered “direct carcinogens” as they can directly interact with biological macromolecules without metabolic activation (Luch, 2005). Most chemical carcinogens require \textit{in vivo} metabolic activation to form reactive electrophilic intermediates that covalently bind to nucleophilic macromolecules including DNA, RNA and proteins (Heidelberger, 1977; Luch, 2005; Miller and Miller, 1981). These chemicals include aromatic and heterocyclic amines, aminoazo dyes, polyaromatic hydrocarbons, \(N\)-nitrosamines and halogenated olefins (Luch, 2005).

1.2.2 Tumour promotion

The promotion stage of carcinogenesis involves the selective clonal expansion of the initiated cell by long term exposure to promoting agents. This stage is considered to be reversible and dose-dependent (Klaunig and Kamendulis, 2004). Compared to initiation, direct changes to DNA structure or genetic code are not observed during promotion (Pitot \textit{et al}., 1981). Promoting agents (including hormones, drugs, metabolites or other chemicals) bind to surface, cytoplasmic or nuclear receptors of the initiated cell, induce changes in transcription and alter gene expression (Pitot \textit{et al}., 1981). For example, chemical compounds such as benzo[\(a\)]pyrene (BP) and 2,3,7,8-tetrachlorodibenzo-\(p\)-dioxin (TCDD) can act as tumour promoters through arylhydrocarbon receptor-mediated signal transduction. Both chemicals can bind arylhydrocarbon receptors in the
cytoplasm, dimerize and translocate to the nucleus where the complex bind DNA response elements and regulate expression of several key proteins. Genes that promote growth and differentiation are upregulated while genes that promote apoptosis are downregulated (Luch, 2005). BP and TCDD can also alter expression patterns of cell cycle proteins such as cyclins via arylhydrocarbon receptors. Taken together, transcriptional changes in factors that control growth, differentiation, apoptosis and cell cycle ultimately result in the clonal expansion of the initiated cells.

1.2.3 Tumour progression

Progression is the final stage of carcinogenesis and occurs when the preneoplastic cells are converted into cancer cells. The progression stage is irreversible and cells undergo karyotypic change characterized by aneuploidy and chromosomal instability that can be observed by light microscopy (Pitot et al., 1981). A loss of genetic integrity results in increased growth rate, increased invasiveness, and changes in biochemical and morphological characteristics that drives formation of the primary tumour.

1.3 Lung cancer and smoking

Globally lung cancer is the leading cause of cancer death among men and second leading cause of cancer death among women, accounting for 1.5 million new cases and 1.3 million deaths annually (World Health Organization, 2010). In Canada, lung cancer is the leading cause of cancer-related death and the second most diagnosed cancer among men and women (Canadian Cancer Society, 2010). Lung cancer was diagnosed in 24,000 Canadians and claimed over 20,000 lives in 2010 (Canadian Cancer Society, 2010). Once diagnosed, the 5-year prognosis of lung cancer is poor with only 10 to 15% of patients surviving (Sekido et al., 1998; Wood et al., 2004).
An extensive amount of epidemiologic data collected in the past 50 years has clearly established the link between cigarette smoking and lung cancer. It is estimated that smoking and exposure to tobacco smoke cause 85 to 90% of lung cancer cases in men and 75 to 80% of lung cancer cases in women (Hecht, 1999; Subramanian and Govindan, 2007). Cigarette smoke is chemically complex and currently contains over 4000 chemicals, 50 that are known carcinogens including polycyclic aromatic hydrocarbons, aromatic amines and tobacco specific nitrosamines (Health Canada, 2010). The particulate phase of cigarette smoke contains over 3500 compounds including carcinogens (Hecht, 1999). Unburned tobacco contains at least 16 known carcinogens (Hecht, 2003) and smokeless tobacco products that are rising in popularity and are perceived as a safer alternative to cigarettes, contain 30 known carcinogens (Boffetta et al., 2008). It should be noted that cigarettes and tobacco smoke can also increase the risk for other serious health problems including heart disease, stroke and chronic lung disease. They have also been linked to cancer of the larynx, oesophagus, mouth, bladder, cervix, pancreas, and kidneys (World Health Organization, 2010).

Since the mid 1950s there has been a decline in smoking prevalence in Canada and other developed countries like the US and UK (Giovino, 2002). In 1985, 35% of Canadians aged 15 and older were considered current smokers, but by 2008 the rate dropped to 15% (Health Canada, 2010). The decline in smoking prevalence in Canada is due to a combination of factors including tax increases, advertising restrictions, prominent health warnings, smoking restrictions in public places and educational campaigns over the past 60 years (Cunningham, 1996).

Even though smoking rates have declined, in recent years the drop has started to level off suggesting that a proportion of the population (approximately 4.9 million Canadians) will continue to smoke cigarettes despite increased efforts to curb the behaviour (Health Canada, 2010). These individuals are at an increased risk of developing lung cancer compared to former
smokers and never-smokers (Sekido et al., 1998). In addition, individuals who are never-smokers but are exposed to tobacco smoke from direct smoking exhalation (mainstream smoke) or an idle burning cigarette (sidestream smoke) have an increase risk of lung cancer (Hecht, 1999). Never-smokers are also exposed to carcinogens and particulate matter from cigarette smoke that have been trapped in hair, skin, and fabrics (Health Canada, 2010). Former smokers are also at risk of lung cancer as smoking cessation reduces the risk, but cancer risk will never return to levels of never-smokers (Sekido et al., 1998). Although the decline in smoking rates in developed countries like Canada are encouraging, the global statistics offer a stark reality of tobacco use.

The World Health Organization (WHO) has termed the global use of cigarettes an epidemic. There are currently more than 1 billion smokers with nearly 80% of them living in low- and middle-income countries (World Health Organization, 2010). Global tobacco consumption is currently increasing 3.4% each year. Cigarettes are widely available and accessible with 5.5 trillion cigarettes being produced and consumed annually (World Health Organization, 2010). More than 5 million people die per year from tobacco use and by 2030 this number is expected to rise to 8 million people (World Health Organization, 2010). Hence, tobacco use is the leading cause of preventable premature death. Large epidemics of lung cancer are expected in China and India where approximately 40% of all smokers reside (Hecht, 1999). In China it is estimated that several million cases of lung cancer will arise by 2050 based on current smoking prevalence and population growth (Liu et al., 1998).

Although smoking and exposure to tobacco smoke are responsible for the majority of lung cancer cases, genetic variation likely plays a role in pulmonary carcinogenesis. Only 20% of smokers actually develop lung cancer, suggesting that some individuals may have genetic susceptibility towards carcinogenesis (Wei and Spitz, 1997). A likely gene candidate that may contribute to familial lung cancer and lung cancer susceptibility is RGS17 found on chromosome
6q23-25 (You et al., 2009). The RGS17 protein is a member of the regulator of G-protein signaling (RGS) family that can indirectly enhance or inhibit the activities of downstream proteins (e.g. adenylate cyclase) and secondary messengers (e.g. cyclic AMP) (You et al., 2009). Lung tumours from cancer patients have been shown to accumulate high levels of RGS17 mRNA transcripts compared to normal lung tissue, suggesting a potential role in carcinogenesis (You et al., 2009). Other factors such as genetic variation in an individual’s ability to metabolize carcinogens into genotoxic intermediates, detoxify those intermediates, and repair damaged DNA can also determine susceptibility to carcinogenesis. Polymorphisms in genes encoding proteins that participate in such reactions are associated with lung cancer risk (Schwartz, 2004).

1.4 Human lung cancer classification

The lung is comprised of over 40 unique cell types that perform a variety of functions such as gas exchange at the alveoli, blood-barrier at the alveolar junction, immunity against pathogens and removal of particulate matter from the respiratory tract. In 98% of lung cancer cases the epithelial cells lining the bronchiolar airway of the lung are transformed into cancerous cells called carcinomas (Travis et al., 2004). Lung cancer has heterogeneous tumour biology compared to other common cancers such as breast, colon and prostate (Borczuk et al., 2003). It is histologically categorized into two major classes; small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). Other rare tumour types include sarcomatoid carcinoma, salivary gland tumours and carcinoid tumours (Travis et al., 2004).

SCLC represent 15 to 25% of all lung cancer cases with tumours centrally located in larger airways such as the primary or secondary bronchi (Sekido et al., 1998). Compared to normal cells of the bronchi, SCLC cells are small and have scant cytoplasm, ill defined cell borders, finely granular chromatin, and inconspicuous or absent nucleoli (Jackman and Johnson,
SCLC is an aggressive form of cancer that has rapid replication rate and undergoes early metastasis (Wood et al., 2004). Although most individuals’ SCLC initially respond to chemotherapy and radiation therapy, prognosis is poor with 3% survival over 3 years (Chute et al., 1999). SCLC occurs in individuals with smoking history and has a strong association with tobacco use (Hanrahan and Glisson, 2010).

Non-small cell lung cancer (NSCLC) consist of 75 to 85% of lung cancer cases and are further divided into major subtypes; squamous cell carcinoma (SCC) (30% of all lung cancer cases), adenocarcinoma (AC) (40%) and large cell carcinoma (9%) (Sekido et al., 1998). Development of SCC and AC are commonly associated with smoking. SCC originate in the central bronchus while AC are found in peripheral lung, in distal airways and alveoli of the lung (Corvalan and Wistuba, 2010).

1.4.1 Modelling lung cancer in mouse

Lung cancers in both mice and humans share morphologic, histogenic and molecular features. Some strains of mice are particularly more sensitive to chemical induction of lung cancer from tobacco specific carcinogens or cigarette smoke (Meuwissen and Berns, 2005). The A/J and SWR mice are the most sensitive mouse strains, while others range from intermediate sensitivity (O20, BALB/c) to more resistant (CBA, C3H) and highly resistant (C57BL/6 and DBA) (Meuwissen and Berns, 2005). Several pulmonary adenoma susceptibility loci have been mapped to oncogenes and tumour suppressor genes and can be attributed to sensitivity between strains of mice. For example, polymorphisms in intron 2 of the K-ras oncogene are found in most susceptible strains of mice and are thought to induce major increases in K-ras expression as well as alter sensitivity to respiratory carcinogens (You et al., 1993; Chen et al., 1994). The K-ras protein is a GTPase that activates and recruits other proteins in signal transduction pathways to
facilitate growth of cells. Polymorphisms also occur at higher frequency in sensitive mice at a susceptibility locus on chromosome 4 that encodes the tumour suppressor gene $p16^{ink4a}$ ($p16$) (Zhang et al., 2002; Herzog et al., 1999). The p16 protein is critical in regulating progression in the cell cycle and in particular G1/S cell cycle transition. Mutations in $K-ras$ and $p16$ gene have also been well documented in human SCLC and NSCLC, suggesting development of lung cancers between mice and humans could occur in a similar fashion (Sekido 1998).

The development of AC typically involves a series of morphologically distinct preneoplastic changes (Horio et al., 1996). They include the sequential stages of hyperplasia, adenoma, carcinoma in situ and finally metastatic carcinoma in both mice and humans (Stoner, 1998; Malkinson, 1998). In mouse models, the induction of lung tumours with chemical carcinogens typically results in adenomas and ACs. One way in which mice and humans differ is that malignant AC rarely metastasize in mouse models, as mice usually die from respiratory distress caused by carcinoma masses in lung that obstructs gas exchange (Malkinson, 1998). The cause of death in human AC patients is usually related to consequences of metastasis (Malkinson, 1998).

1.5 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)

$N$-nitrosamines (referred to nitrosamines in this thesis) are present in many common consumer items including tobacco, cosmetics, beer and bacon (Bertram, 2000). Over 200 nitrosamines and their carcinogenic activity have been confirmed in laboratory animals (Hecht, 1999). Among consumer products, cigarettes and smokeless tobacco contain levels of carcinogenic nitrosamines 100 to 1000 times greater than other products intended for consumption (Hecht, 2003). A major class of nitrosamines found in cigarettes are tobacco
specific nitrosamines (TSNA) (Figure 1.1). 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone is a nicotine derived nitrosaminoketone (NNK) and considerable research indicates that it is likely to be an important contributor to tobacco-induced cancers. Among TSNA characterized, NNK, NNN and NAT are found in greatest quantities in cigarettes and NNK, NNN and NNAL are the most carcinogenic TSNA (Hecht, 1998; Hecht, 2003). Typical cigarettes contain 1 to 2 μg of NNK for every gram of unburned tobacco (Hecht, 2002). Every gram of commonly used smokeless tobacco contains 1 to 5 μg of NNK and NNN (Boffetta et al., 2008).

TSNA are formed from nitrosation of different alkaloids during curing, aging, fermentation and burning of tobacco (Spiegelhalder and Bartsch, 1996; Brunnemann et al., 1996). Nicotine, the addictive component of cigarettes, is the major alkaloid found in tobacco comprising 85 to 90% of total alkaloid content (Boffetta et al., 2008). NNK, NNAL and NNN are specifically formed when nicotine undergoes a nitrosation reaction. The formation of TSNA is dependent on nitrosating agents, such as nitrites (NO\textsubscript{2}^{-}), that can originate from different sources. Nitrate (NO\textsubscript{3}^{-}), a common constituent in tobacco, can be reduced to nitrite which can then specifically react with tobacco alkaloids forming TSNA (Spiegelhalder and Bartsch, 1996). The formation of some TSNA has also been shown to occur in vivo in humans and experimental animals (Osterdahl and Slorach, 1988; Carmella et al., 1997).

1.5.1 NNK and lung cancer

NNK is a potent systemic lung carcinogen found in unburned tobacco and tobacco smoke which induces lung tumours in commonly used rodent models regardless of route of administration. Tumours of the respiratory system have been observed in multiple studies using NNK-treated mice, rats and hamsters. For example, female A/J mice treated with a total dose of
Figure 1.1 Formation of tobacco specific nitrosamines (TSNA) from major (nicotine) and minor (nornicotine, anabasine and anatabine) tobacco alkaloid precursors (modified from Hecht, 1998). Currently, 7 TSNA have been identified including 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), N'-nitrosonornicotine (NNN), N'-nitrosoanabasine (NAB), N'-nitrosoanatabine (NAT), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), 4-(methylnitrosamino)-4-(3-pyridyl)-1-butanol (iso-NNAL), and 4-(methylnitrosamino)-4-(3-pyridyl)butyric acid (iso-NNAC). With the exception of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanal (NNA), all have been detected in tobacco. Note, NNA has only been synthesized and detected in vitro, and therefore technically is not a TSNA (Hecht et al., 1978; Hecht, 1998).
0.11 mmol of NNK i.p., induced an average of 36.7 lung tumours per mouse (Castonguay et al., 1983). In contrast, a similar dose of NNN and NNAL induced only 1.6 and 26.3 lung tumours per mouse respectively. Similarly, among strain A mice treated with different TSNA, NNK induced more lung adenomas per mouse than either NNN or NNA (Hecht et al., 1978). F344 rats treated subcutaneously with a total dose of 3.4 mmol NNK over 20 weeks developed tumours in the nasal cavity, liver and lungs, while the same dose of NNN only gave rise to tumours in the nasal cavity (Hecht et al., 1980). Additionally, Syrian golden hamsters treated with low dose of NNK (5 μmol) induced respiratory tract tumours in 6 of 20 animals treated (Hecht et al., 1983). The accumulation of animal data indicate that NNK is one of the most potent pulmonary carcinogens among TSNA identified.

NNK has organoselectivity for lung tissue in experimental animals, mainly inducing adenomas and ACs regardless of route of administration (i.e. topically, intraperitoneally, intravenously, subcutaneously, intravesically (into the urinary bladder), or administered through drinking water) (Hoffmann et al., 2001; LaVoie et al., 1987; Prokopczyk et al., 1991; Lijinsky et al., 1991). In addition to being a potent pulmonary carcinogen, treatment with NNK results in cancers of other tissues and organs. Tumours of the nasal cavity, oral cavity and liver occur in a variety of rodents treated with NNK (Hecht and Hoffmann, 1988; Hecht, 2003). Furthermore, NNK and NNAL are the only known pancreatic carcinogens in unburned tobacco and tobacco smoke. Male F344 rats treated with NNK or NNAL in drinking water significantly induced ACs of acinar or ductal origin in the exocrine pancreas (Rivenson et al., 1988). Pancreatic lesions are spontaneous in control animals and rarely observed (Hecht, 2003). NNK could also be responsible for tobacco-induced cervix cancer. Levels of NNK and its metabolites were elevated in the cervix of women who smoke (Prokopczyk et al., 1997). Cervical cells and subcellular
fractions of the cervix have the capacity to bioactivate NNK and could generate reactive intermediates that damage DNA (Prokopczyk et al., 2001).

NNK’s capacity to specifically induce lung adenoma and AC may be important in tobacco-induced lung cancers in humans. The tumours induced by NNK in rodent species are morphologically and topographically similar to human bronchiolar and bronchioalveolar AC (Wynder and Hoffmann, 1994). Among smokers, the incidence of AC has increased over the past 5 decades, surpassing SCC as the most common form of lung cancer. The analysis of lung cancer cases from the late 1960s to the late 1980s has shown an increase in the prevalence and rate of AC cases compared to SCC cases (Devesa et al., 1991; Auerbach and Garfinkel, 1991). Changes in cigarette design and smoking behaviour have likely contributed to the shift in AC. The introduction of filter tips to cigarettes traps the particulate phase of tobacco smoke but also impedes the delivery of nicotine into the body. To compensate for decreased levels of nicotine, cigarette users tend to inhale more deeply, and smoke more intensely to increase pulmonary absorption of nicotine. This practice also disperses tobacco smoke carcinogens farther into the respiratory tract and exposes the distal bronchi and bronchioalveolar junction to relatively high levels of smoke carcinogens, including polyaromatic hydrocarbons, volatile aldehydes and TSNA. Reduction of nicotine levels in low tar cigarettes has also resulted in similar changes in smoking behaviour. Furthermore, the chemical components of cigarettes have been altered and may have contributed to increased AC. Between 1959 and 1997, tobacco blends have incorporated more air cured tobacco, resulting in cigarettes and tobacco products that have higher nitrate and TSNA content, including NNK, while lowering the amount of polyaromatic hydrocarbons (Hoffmann et al., 2001). More intense smoking, deeper inhalation and increased TSNA content in cigarettes are considered to be major contributors in increasing lung AC among smokers (Wynder and Hoffmann, 1994).
1.5.2 Metabolic pathways of NNK

The main NNK metabolism pathways are illustrated in Figure 1.2. To exert carcinogenicity, NNK requires metabolic activation that generates reactive metabolites that can adduct DNA. Bioactivation or metabolic activation refers to the process where an inactive compound is converted into active metabolites. NNK undergoes major reactions including carbonyl reduction, pyridine oxidation, and $\alpha$-hydroxylation. Denitrosation and ADP adduct formation of NNK have only been observed in vitro using rat liver microsomes (Castonguay et al., 1991; Peterson et al., 1994). The carbonyl reduction of NNK, catalyzed by 11$\beta$-hydroxysteroid dehydrogenase, cytosolic carbonyl reductase and aldo-keto reductase, result in the major metabolite NNAL (Maser, 2004). In general, the reduction of NNK to NNAL occurs in many tissues including lung and liver. Upon NNK administration, NNAL is rapidly formed and is the predominate metabolite in blood (Hecht, 1998). NNAL is not a final detoxified product, since it can undergo bioactivation and has carcinogenic activity (Rivenson et al., 1988; Castonguay et al., 1983). The reconversion of NNAL to NNK via oxidation does not occur as extensively as reduction, however it may be of interest as it could play a role in NNAL carcinogenicity.

The $\alpha$-hydroxylation reactions of NNK and NNAL are of major interest as they result in reactive intermediates that can covalently bind DNA, RNA and proteins. Like most carcinogens, NNK and its metabolites require metabolic activation to exert carcinogenic effect. The $\alpha$-hydroxylation occurs on the methyl or methylene carbons that are adjacent to the N-nitroso group. Hydroxylation of the methyl carbon of NNK forms 4-(hydroxymethylnitrosamino)-1-(3-pyridyl)-1-butanone (1), that decomposes to 4-oxo-4-(3-pyridyl)-1-butanediazohydroxide (2) and formaldehyde. Compound (2) is highly reactive and can form bulky pyridyloxobutyl (POB) adducts on DNA, RNA and protein. Compound (2) can also react with water forming 4-hydroxy-
Figure 1.2 Pathways of NNK metabolism. Metabolites I and II are detoxified products produced by pyridine oxidation. Metabolites III to VII are endpoints of $\alpha$-hydroxylation and are generated by reactive metabolites that pyridyloxobutylate $\ominus$, methylate $\star$ and pyridylhydroxybutylate $\star\star$ DNA (modified from Smith et al., 2003).
1-(3-pyridyl)-1-butanone (keto alcohol or HPB) (III). HPB can be quantified to determine the extent of hydroxylation at NNK’s methyl carbon.

Like NNK, NNAL also undergoes similar α-hydroxylation reactions to form electrophilic metabolites. The α-hydroxylation of the methyl carbon of NNAL forms an intermediate (3), which decomposes to formaldehyde and the DNA pyridylhydroxybutylating species, 4-hydroxy-4-(3-pyridyl)-1-butanediazohydroxide (4). Compound (4) can react with water to form 4-hydroxy-1-(3-pyridyl)-1-butanol (Diol) (VI) or cyclizes to 2-(3-pyridyl)tetrahydrofuran (pyridyl-THF) (VII).

Hydroxylation of the methylene carbon of NNK results in the formation of 4-hydroxy-4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (5) that spontaneously decomposes to methane diazohydroxide and 4-oxo-4-(3-pyridyl)butanal (keto aldehyde or OPB) (6). Methane diazohydroxide is a methylating agent and interacts with DNA at several bases. OPB can be trapped as a bisulfite adduct to quantify α-methylene hydroxylation or undergo further oxidation to form 4-oxo-4-(3-pyridyl)butyric acid (IV) (keto acid of OPBA). Hydroxylation at the methylene carbon of NNAL (7), ultimately yields the methylating species, methane diazohydroxide (8) and a hydroxy aldehyde which can cyclize to 5-hydroxy-2-(3-pyridyl)tetrahydrofuran (lactol) and could be further oxidized to 4-hydroxy-4-(3-pyridyl)butyric acid (hydroxy acid) (V).

The major pathway of detoxification of NNK and NNAL occurs via pyridine oxidation. Pyridine N-oxidation of NNK and NNAL generates 4-(methylnitrosamino)-1-(3-pyridyl-N-oxide)-1-butanone (NNK-N-oxide) (I) and 4-(methylnitrosamino)-1-(3-pyridyl-N-oxide)-1-butanol (NNAL-N-oxide) (II) respectively. These N-oxide products are readily excreted through the urine. Glucuronidation of NNAL is another important detoxification route and can occur at the carbinol or the pyridine nitrogen of NNAL (Not shown in Figure 1.2). In addition O-
glucuronidation of compound 2 has been observed in rats and is a potential detoxified product (Murphy et al., 1995).

1.5.3 Cytochrome P450 enzymes and bioactivation of NNK

The metabolic activation of NNK is catalyzed mainly by cytochrome P450 (CYP) enzymes. In particular, the CYP1, CYP2 and CYP3 families of enzyme are primarily responsible for catalyzing oxidation of xenobiotics, generating polar metabolites that can either be excreted or remain within the body for further biotransformation. The liver is the major site of xenobiotic metabolism, containing an abundance of CYP enzymes that are easily accessible for study. The respiratory tract also contains CYPs and can be a site of metabolism for therapeutics and xenobiotics that are inhaled or in the systemic circulation. The pharmacological and toxicological effects of agents depend on the lung’s ability to activate or deactivate these agents via biotransformation and in fact, the carcinogenicity of NNK depends on metabolic activation in the respiratory system. Although human lung tissue contains similar hepatic CYPs involved in xenobiotic metabolism, low level expression of pulmonary CYPs make it difficult to fully characterize their identity and absolute levels. The levels of pulmonary CYPs are not uniform throughout the lung and may vary depending on cell type and region. Enzymes that likely play a major role in NNK hydroxylation in human lung and liver include CYP1A1, 1A2, 2A6, 2A13 and 2B6 (Nademanee, 1992; Smith et al., 1995; Smith et al., 1996; Patten et al., 1996; Su et al., 2000; Jalas et al., 2003; Jalas et al., 2005).

1.5.4 NNK and DNA adducts

The metabolic activation of NNK and NNAL results in reactive metabolites that can methylate and pyridyloxobutylate DNA. The methane diazohydroxide metabolite generated from NNK metabolism, methylates DNA at several bases producing 7-methylguanine (7-mG), O6-
methylguanine (O\textsuperscript{6}-mG), and O\textsuperscript{4}-methylthymine (O\textsuperscript{4}-mT) (Hecht, 1998). NNK-induced pyridyloxobutyl (POB) adducts are bulky DNA adducts and are detected at the N\textsuperscript{7}, N\textsuperscript{2} and O\textsuperscript{6}-positions of guanine and O\textsuperscript{2}-positions of cytosine and thymine (Jalas et al., 2005). The extent of NNK-induced DNA methylation and POB adduction are dependent on a number of factors including the site of hydroxylation (the methyl or methylene carbon of NNK), levels of bioactivation and detoxification, and ability to repair DNA adducts. NNK’s ability to induce both methyl and POB adducts may be important for carcinogenicity as most other nitrosamines just induce one type of DNA damage. Treatment with either NNN or N-nitrosodimethylamine, agents that can only pyridyloxobutylate or methylate DNA respectively, have lower pulmonary carcinogenicity compared to NNK which induce both types of adducts (Hoffmann et al., 1984; Hecht et al., 1986).

Methyl and POB DNA adducts have been detected in the lungs of human smokers. DNA isolated from peripheral lung and tracheobronchial tissue from smokers had significantly higher levels of POB adducts compared to non-smokers (Foiles et al., 1991; Holzle et al., 2007). The 7-mG adduct was found at higher levels in smokers compared to non-smokers (Mustonen et al., 1993). Similarly, O\textsuperscript{6}-mG was also detected in the lungs of smokers (Wilson et al., 1989). It should be noted that methyl and POB adducts detected in smokers could be due to reactive metabolites generated from NNK, but also from other nitrosamines including NNN and N-nitrosodimethylamine found in cigarettes and tobacco smoke (Hecht, 2003).

The O\textsuperscript{6}-mG adduct may be of particular importance in pulmonary carcinogenesis in mice. O\textsuperscript{6}-mG is strongly correlated with tumour multiplicity in mice treated with NNK or acetoxymethyl-methylnitrosamine, an analog of NNK that exclusively methylates DNA (Peterson et al., 2001). In addition, O\textsuperscript{6}-mG adducts that are persistent and escape DNA repair, induce G→A transition mutations in DNA. GGT→GAT mutations have been found in codon 12 of the
K-ras oncogene in a high proportion of lung AC induced by NNK in A/J mice, suggesting a possible role of O\textsuperscript{6}-mG in tumour induction (Belinsky et al., 1989). Persistent POB adducts can also induce mutations including G\rightarrow A and G\rightarrow T in codon 12 of K-ras gene in lung tumours of NNK-treated mice (Ronai et al., 1993). Mutations at codons 12, 13 and 61 of K-ras can result in uncontrolled growth and potentially develop into cancers. K-ras mutations are detected in 20 to 30% of human lung ACs and 15 to 20% of all non-small cell lung cancers, with frequent mutations at codon 12 (Sekido et al., 2003). Lung AC from smokers frequently had more K-ras mutations compared to ACs from former smokers and never-smokers (Slebos et al., 1991; Westra et al., 1993).

NNK has also been shown to induce epigenetic changes by altering methylation status of the p16 tumour suppressor gene. The specific methylation of cytosine residues in CG dinucleotides that are found near the promoter region of genes, can silence expression via chromatin remodelling. For example, in vivo treatment with NNK in rats induced hypermethylation in CG dinucleotides in the promoter region of the p16 gene in 94% of lung AC (Belinsky et al., 1998). Methylation of the promoter region was associated with reduced p16 protein levels that could impair cell cycle checkpoints and potentially contribute to carcinogenesis (Belinsky et al., 1998). In vivo treatment with NNK is likely altering the activity of DNA methyltransferases that are responsible for methylating cytosine residues in CG dinucleotides (Belinsky, 1998).

1.6 Base excision repair and oxidative stress

An organism’s genome is vulnerable to a wide array of DNA damaging agents of endogenous and exogenous origin. DNA repair systems are required to maintain genomic integrity by identifying and removing damage from the DNA helix structure to prevent
mutagenicity and cytotoxicity. Several types of DNA repair have been identified including base excision repair (BER), mismatch repair, nucleotide excision repair (NER) and direct reversal repair. Each type of repair is responsible for fixing specific types of DNA damage.

The BER pathway recognizes and replaces a wide array of damaged bases from DNA. A major function of BER is to remove nucleotides that have been oxidized by reactive oxygen species (ROS). ROS is a collective term that can refer to a free radical oxygen species that have an unpaired electron in its valence shell; it also refers to certain non-radicals that are either strong oxidizing reagents or easily converted to free radicals (Wiseman and Halliwell, 1996). The major ROS generated in biological systems include superoxide radical anions (O$_2^-$), hydroxyl radicals (●OH), peroxyl radicals (RO$_2$●), alkoxy radicals (RO●), hydrogen peroxide (H$_2$O$_2$) and singlet oxygen (‘O$_2$) (Wiseman and Halliwell, 1996). Production of ROS can arise from both endogenous and exogenous sources. Different environmental agents including tobacco smoke and air pollutants contain ROS and can generate ROS. Chemical carcinogens (such as benzo[α]pyrene, aflatoxin and benzene), ionizing radiation and UV light can also induce ROS (Loft and Poulsen, 1996).

In biological systems, a major source of endogenous ROS is the complete reduction of oxygen via the electron transport chain in mitochondria during cellular respiration. Approximately 4 to 5% of molecular oxygen that undergoes one-electron reduction is converted to superoxide radical anions (Klaunig and Kamendulis, 2004). Mitochondrial superoxide dismutase, an antioxidant enzyme, can catalyze the conversion of excess superoxide radical anions to hydrogen peroxide (Klaunig and Kamendulis, 2004) while catalase and glutathione peroxidase convert hydrogen peroxide to water. Excess hydrogen peroxide can leak from the
mitochondria and into the cytosol (Yu, 1994). In the presence of ferrous iron, hydrogen peroxide can decompose to hydroxyl radicals via the Fenton reaction:

\[
Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \cdot OH + OH^-
\]

Hydroxyl radicals are also formed in a similar manner when reduced forms of other transition metals such as copper come into contact with hydrogen peroxide. Iron and copper are sequestered in proteins including ferritin, transferrin, caeruloplasmin and metallothionein. Oxidative stress has been shown to release transition metals from proteins and the released metals can then participate in Fenton reactions to produce hydroxyl radicals (Halliwell and Aruoma, 1991).

Numerous enzymes that catalyze redox reactions can also contribute to the generation of ROS. During CYP catalyzed metabolism, the generation of ROS can arise via different processes. Redox cycling in the presence of molecular oxygen and uncoupling can result in the generation of superoxide anion radical and hydrogen peroxide (Klaunig and Kamendulis, 2004). Several CYPs have the ability to generate ROS during metabolism. CYP2E1 produces a prolonged burst of ROS near the site of substrate oxidation during ethanol metabolism (Ekstrom and Ingelman-Sundberg, 1989). Similarly, the metabolism of phenobarbital by CYP2B can uncouple resulting in release of superoxide radical anion (Rice et al., 1994). Other enzymes can also produce ROS when catalyzing reactions. Organelles derived from endoplasmic reticulum called peroxisomes are responsible for breaking down fatty acid chains and contain high levels of oxidase enzymes that generate hydrogen peroxide (Yu, 1994). The immune response can generate excess ROS in a process called the respiratory burst. Activated macrophages and hepatic Kupffer cells elicit rapid and transient increase in oxygen uptake to generate superoxide anion radicals and hydrogen peroxide (Klaunig and Kamendulis, 2004). Release of ROS is
important for a host’s defense and results in localized tissue inflammation and induction of genotoxicity and cytotoxicity in foreign microbes (Yu, 1994).

### 1.6.1 Genetic damage induced by ROS

Excess production of ROS can shift cells into an oxidative state with deleterious consequences to DNA. In a given cell, an estimated $10^5$ oxidative DNA lesions are formed per day (Fraga et al., 1990). A wide variety of modifications on pyrimidine and purine bases as well as the sugar-phosphate backbone in DNA have been observed in the oxidative state. In addition, ROS can directly and indirectly result in single or double stranded DNA breaks and abasic sites. Although over 20 base lesions have been identified, the most abundant lesion detected is the hydroxylation of C-8 in guanine residues known as 8-hydroxydeoxyguanosine (8-OHdG) (Cooke et al., 2003). Oxidation of guanine can also result in the ring opened product 2,6-diamino-4-hydroxy-5-formamidopyrimidine. Other abundant lesions of DNA oxidation include 8-oxoadenine, 2-hydroxyadenine, 5-hydroxycytosine, cytosine glycol and thymine glycol (Loft and Poulsen, 1996). The type of oxidative lesion generated depends on the type of ROS interacting with DNA. Superoxide anion radical and hydrogen peroxide have low reactivity with DNA directly (Wiseman and Halliwell, 1996). However, hydroxyl radicals are highly reactive and can induce almost any type of oxidized DNA modification (Loft and Poulsen, 1996). Singlet oxygen generated by UV light has high specificity for guanine residues resulting in 8-OHdG.

If left unrepaired, oxidative DNA damage can induce miscoding mutations in DNA. The vast majority of oxidative lesions result in base substitutions such as transition or transversion mutations. A transition mutation is a point mutation that changes purine nucleotides into the opposite purine (A→G) or pyrimidine nucleotides into the opposite pyrimidine (C→T). Conversely, a transversion mutation refers to substitution of a pyrimidine for a purine or vice
versa. Base deletions and insertions are seldom observed as a result of oxidized DNA (Loft and Poulsen, 1996).

Although ROS can modify all DNA bases, modifications of GC pairs are common in the oxidized state. Hydroxyl radicals from ionizing radiation can induce potential GC→CG or AT base pair substitutions (Loft and Poulsen, 1996). Excess singlet oxygen can react with guanine and induce GC→AT base pair substitutions (McBride et al., 1992; Epe, 1991). ROS can also interact with the deoxynucleotide pool resulting in oxidized nucleotides that can be misincorporated in DNA during synthesis. For example, oxidized deoxyguanosine triphosphate (dGTP) from the nucleotide pool can misincorporate opposite adenine (Cheng et al., 1992).

8-OHdG, a major oxidative lesion, is mutagenic in bacterial and mammalian cells that can produce a G→T transversion in oncogenes and tumour suppressor genes (Klaunig and Kamendulis, 2004; Loft and Poulsen, 1996). 8-OHdG has been shown to induce mutations in codon 12 of the K-ras oncogene in murine fibroblast cells (Jackson, 1994). Human fibroblasts exposed to ROS generating systems induced G→T and C→A transversions at codon 249 of the p53 tumour suppressor gene, an important mutation commonly seen in human tumours (Hussain et al., 1994). Oxidative DNA damage has also been shown to induce transformation of normal cells. Syrian hamster embryos treated with ROS generating systems developed 8-OHdG lesions and underwent cellular transformation (Zhang et al., 2000). Antioxidants inhibited the formation of 8-OHdG and cellular transformation in cells that were treated with ROS.

1.6.2 NNK and DNA oxidation

NNK has been shown to induce oxidative DNA damage in rodent species. F344 rats treated with a single i.p. dose of NNK (20 mg/rat) had significantly higher 8-OHdG levels in lung compared to controls (Chung and Xu, 1992). Treatment of female A/J mice with multiple doses
of NNK (0.25 mg/mouse, 3 times weekly for 3 weeks by gavage) caused significantly elevated levels of 8-OHdG in lung and liver DNA (Chung and Xu, 1992). Similarly, female A/J mice treated with multiple doses of NNK via gavage (0.5 mg/mouse, 3 times per week for 3 weeks) or a single dose of NNK i.p. (2 mg/mouse) had elevated levels of 8-OHdG in lung DNA (Rosa et al., 1998). The use of antioxidant agents in conjunction with NNK, also suggests NNK can induce oxidative DNA damage. Concomitant treatment with (–)-epigallocatechin-3-gallate, an antioxidant found in green teas, significantly reduced the number of lung adenomas per mouse and 8-OHdG levels in lungs of A/J mice chronically treated with NNK via gavage (Xu et al., 1992). In the same study, (–)-epigallocatechin-3-gallate exerted little effect on levels of O⁶-mG in NNK-treated mice, indicating a potential role of oxidation in carcinogenesis (Xu et al., 1992).

Oxidation of DNA can also result in other forms of genetic damage, such as DNA strand breaks. Human lung fibroblasts incubated with NNK resulted in a dose dependent increase in DNA strand breaks (Weitberg and Corvese, 1993). Co-treatment with NNK and hypoxanthine/xanthine oxidase, enzymes that generate ROS, further increased the levels of strand breaks compared to NNK alone. Conversely, concomitant treatment with NNK and oxygen radical scavengers such as mannitol, catalase and superoxide dismutase protected DNA from strand breaks, suggesting NNK can potentiate its effects through ROS (Weitberg and Corvese, 1993). Treatment with NNK can also induce micronuclei in rat fibroblast skin cells in a dose dependent manner. Formation of micronuclei occurs when DNA fails to divide equally between daughter cells during cell division due to DNA strand breaks by exogenous agents such as chemicals or radiation. Superoxide dismutase, an antioxidant enzyme, reduced micronuclei formation in rat skin fibroblasts treated with NNK, suggesting involvement of NNK mediated ROS production in DNA strand breakage (Kim and Wells, 1996).
The mechanism by which NNK induces oxidative DNA damage has not been fully characterized. It is hypothesized that ROS can be generated during CYP metabolism of NNK. During the first step of oxidation, iron(III) of the protoporphyrin-IX ring of a CYP creates a bond with triplet oxygen. The iron(III)-oxygen complex is relatively stable; however superoxide anion radicals (O$_2^-$) can be released, resulting in the breakdown or uncoupling of the oxidation reactions catalyzed by CYP (Meunier et al., 2004). Additional superoxide anion radicals and electrons can also be released during electron transfers in the CYP catalytic cycle. Microsomes containing human CYP1A1, CYP1A2, CYP2B6 and CYP3A4 have been shown to produce superoxide anion radicals during substrate metabolism (Puntarulo and Cederbaum, 1998). A proposed mechanism of generation of ROS during CYP metabolism of NNK is outlined in Figure 1.3. This involves uncoupling during CYP metabolism of NNK, resulting in release of superoxide anion radical. Although the mechanism has not been fully characterized, it appears that NNK can generate ROS that can oxidize DNA.

1.7 Base excision repair (BER) pathway

Repair of oxidative damage to the genome by BER is carried out in three major steps: recognition and excision of the damaged DNA bases, insertion of nucleotides and finally ligation (Figure 1.4). The first step of BER is carried out by DNA glycosylases that recognize and remove damaged or incorrect bases by hydrolyzing the N-glycosidic bond between base and deoxyribose sugar (Christmann et al., 2003). In human cells, 11 different glycosylases have been identified and characterized based on the types of modified bases removed (Christmann et al., 2003). For example, 8-oxoguanine glycosylase 1 (OGG1) recognizes and releases 8-OHdG paired with cytosine. Adenine-DNA glycosylase (MUTYH), identifies and removes 8-OHdG lesions that have incorrectly mispaired with adenine during DNA replication in humans.
**Figure 1.3** Proposed mechanism of generation of ROS by uncoupling of CYP during NNK metabolism (modified from Yang and Smith, 1996). During α-hydroxylation of NNK, the initial oxidation step is believed to form an α-nitrosamino radical and superoxide anion at the CYP iron(III) protoporphyrin-IX ring (initial radical species not shown in figure). In some cases both radical species recombine, in a process called oxygen rebound to form the alcohol intermediate (1). In other cases uncoupling could occur and release superoxide anion from the CYP active site. The resulting α-nitrosamino radical (2) could fragment into nitric oxide (3) and an imine (4). Nitric oxide could then be oxidized to nitrite (NO$_2^-$) while the imine could be hydrolyzed to an aldehyde (5) and methylamine (6).
Figure 1.4 Mechanisms of BER pathway depicting short patch and long patch repair (Christmann et al., 2003). AP: apurinic/apyrimidinic; 5’dRP: 5’-deoxyribose-5-phosphate; Polβ, Polδ/ε: DNA polymerases; Lig I, Lig III: DNA ligases; XRCC1: X-ray repair cross-complementing protein 1; RF-C: replication factor C; Fen1: flap endonuclease 1; PCNA: proliferating cell nuclear antigen.
Glycosylases have been further divided into 2 major subtypes; type I and type II. Type I glycosylase identify and remove modified bases leaving an apurinic or apyrimidinic site (AP) in DNA (Christmann et al., 2003). Type II glycosylases recognize and remove damaged bases, but can also cleave the DNA phosphodiester backbone by endogenous 3’-endonuclease activity, giving rise to a single strand break (Christmann et al., 2003). In the case of type I glycosylases, the phosphodiester backbone of DNA is cleaved by a separate AP endonuclease that can result in 5’-deoxyribose-5-phosphate (5’dRP) and 3’-hydroxyl DNA termini (Fortini and Dogliotti, 2007).

After removal of the damaged base, depending on the type of BER, a single nucleotide or series of nucleotides will be inserted. In short patch repair, DNA polymerase β (Polβ) exerts lyase activity and catalyzes the release of the hemiacetal form of 5’dRP formed at the DNA terminus of the AP site (Christmann et al., 2003). The resulting gap is filled by a single nucleotide by Polβ. DNA ligase III (Lig III) participates in short patch repair and seals the DNA backbone (Christmann et al., 2003). Both Lig III and Polβ have been shown to bind with the scaffolding protein X-ray repair cross-complementing protein 1 (XRCC1) for added stability and function (Caldecott et al., 1994; Robertson et al., 2009). The net result of short patch repair is replacement of the damaged base by a single nucleotide.

In long patch repair, Polβ first inserts a single nucleotide at the AP site (Fortini and Dogliotti, 2007). After Polβ dissociation, further DNA synthesis is done by DNA polymerase δ and ε, resulting in a longer repair patch (Christmann et al., 2003). Proliferating cell nuclear antigen (PCNA) and replication factor C (RF-C) are recruited to stabilize DNA polymerase δ and ε during nucleotide insertion (Stucki et al., 1998). The original 5’dRP flap structure is cleaved by flap endonuclease I (FenI) (Klungland and Lindahl, 1997). PCNA forms a complex with FenI to
stimulate its endonuclease activity. Finally, DNA ligase I interacts with PCNA and Polβ to seal the DNA backbone (Srivastava et al., 1998; Christmann et al., 2003). The net result of long patch repair is replacement of the damaged base by a series of nucleotides, 2 to 15 nucleotides in length.

Other accessory proteins may have a role during BER. The addition of purified p53 protein to cell free extracts or reconstituted BER pathway stimulates BER in vitro. Conversely, immunodepletion of p53 from cell extracts and deficient p53 cell lines have decreased BER activity (Zhou et al., 2001; Achanta and Huang, 2004). The p53 protein has been shown to directly bind and stabilize AP endonuclease and OGG1 glycosylase to enhance BER activity (Achanta and Huang, 2004). Furthermore, p53 has been shown to stabilize the interaction between Polβ and DNA termini at AP sites (Zhou et al., 2001).

1.7.1 Decision between short and long patch repair in BER

The decision to undergo short or long patch BER is not fully characterized. Several hypotheses exist attempting to elucidate the switch between short and long patch repair. The capacity to cleave the 5’dRP terminus by Polβ plays an important role in determining which pathway BER performs. Polβ can cleave the 5’dRP terminus in hemiacetal form and completes BER via the short patch pathway. Oxidized or reduced AP sites, 3’-unsaturated aldehydes or 3’-phosphates are resistant to Polβ lyase activity and result in further processing through the long patch repair pathway (Nakamura et al., 2000; Robertson et al., 2009; Christmann et al., 2003). The type of DNA termini produced at the AP site depend on the oxidative DNA lesion present as well as the glycosylase used for repair (Fortini et al., 1999). Other factors such as relative concentration of ATP and the stage of the cell cycle may also play a role in determining whether long or short patch BER proceeds (Petermann et al., 2003; Fortini and Dogliotti, 2007).
Although short and long patch BER most likely occur concurrently, the degree to which each occurs has not been fully characterized. In vitro assays using cell free extracts suggest that repair of 8-OHdG adducts is carried out predominantly by short patch repair (Dianov et al., 1998; Fortini et al., 1999). However, in intact cells transfected with a plasmid containing an 8-OHdG lesion, repair patches longer than one nucleotide were observed in 55 to 80% of plasmids, suggesting that long patch repair of 8-OHdG also plays a role in intact cell systems (Sattler et al., 2003).

1.7.2 BER and carcinogenicity

Deficient DNA repair can result in persistent DNA damage. If replication occurs before the lesion is repaired, a permanent change in DNA may be introduced, which in turn could potentially contribute to carcinogenesis. Knockout mice have been used to test the effects of deficient BER on oxidative DNA damage and cancer susceptibility. Mice deficient in major glycosylases had no drastic changes on phenotype and marginal effects on spontaneous tumourigenesis. For example, OGG1 knockout mice developed a significantly higher number of spontaneous lung adenocarcinoma (AC) and accumulated higher levels of 8-OHdG lesions in liver compared to wild type mice (Sakumi et al., 2003). Mice deficient in MUTYH glycosylase had increased incidence of spontaneous intestinal tumours (Sakamoto et al., 2007). Knockout mice deficient in both OGG1 and MUTYH accumulated 8-OHdG lesions in lung and small intestine, and had increased frequency of lung and ovarian tumours and lymphomas (Xie et al., 2004; Russo et al., 2004). Although these studies have shown moderate effect on spontaneous tumourigenesis, exposing knockout mice to pro-oxidants can exacerbate carcinogenesis. Treatment with the pro-oxidant dimethylarsinic acid in OGG1-deficient mice resulted in a greater number of lung tumours (Kinoshita et al., 2007). These types of experiments demonstrate that BER can function as a protective mechanism during carcinogenesis.
Human cancers have also been analyzed for mutations in major proteins of the BER pathway. Low OGG1 activity is associated with higher risk of some cancers. Squamous cell carcinoma (SCC) of the head and neck was significantly associated with low OGG1 activity in peripheral blood mononuclear cells (Paz-Elizur et al., 2006). Furthermore, leukocytes and peripheral blood mononuclear cells obtained from lung cancer patients had reduced capacity to repair 8-OHdG lesions in DNA constructs compared to healthy controls (Paz-Elizur et al., 2003). Smokers with low OGG1 activity also had higher risk of developing lung cancer (Paz-Elizur et al., 2003). Low OGG1 activity in cancer patients could be due to polymorphisms, differences in expression levels, protein stability, post-translational modifications, or presence or absence of natural activators and inhibitors.

Deficiency in the MUTYH glycosylase has been linked to rare forms of familial colorectal cancer in humans. A British family with a history of colorectal cancer had a germline biallelic missense mutation in the MUTYH gene that resulted in 2 variants, both with a single amino acid substitution (David et al., 2007). An in vitro study using the human MUTYH variants in E. coli deficient in MutY (homologue of human MUTYH), found decreased repair of 8-OHdG mispaired with adenine compared to wild type MUTYH (Chmiel et al., 2003). Tumours from the family contained G\(\rightarrow\)T transversion mutations in the tumour suppressor gene adenomatous polyposis coli, likely due to the accumulation of oxidative DNA lesions that could not be repaired as a result of deficient BER (David et al., 2007). Thus far, this is the only BER germ line mutation to be directly implicated in carcinogenesis (Paz-Elizur et al., 2008).

While deficient BER can result in elevated oxidative DNA damage and incidence of cancer, conflicting evidence suggests that the mere presence of oxidative DNA damage may not be necessary or sufficient to induce carcinogenesis in vivo (Cooke et al., 2003). Elevated oxidative DNA damage is seen in several human pathological conditions (e.g. rheumatoid
arthritis, systemic lupus, and neurodegenerative conditions) without increase in cancer susceptibility (Cooke et al., 2003) and glycosylase knockout mice did not show increased incidence of spontaneous carcinogenesis in several organ systems (Klungland et al., 1999; Minowa et al., 2000; Paz-Elizur et al., 2008). Other DNA repair systems and redundant glycosylases may be able to mitigate the effect of deficient BER. Mismatch repair and nucleotide excision repair have the limited ability to repair 8-OHdG adducts which could act as a backup repair mechanism in glycosylase knockout mice (Colussi et al., 2002; Reardon et al., 1997). Epidemiologic studies have also shown polymorphisms of major BER genes (OGG1 glycosylase, AP endonuclease, XRCC1, etc.) are weakly associated with cancer and may only play a minor role in carcinogenesis in humans (Hung et al., 2005a; Hung et al., 2005b; Vogel et al., 2004; Paz-Elizur et al., 2008). Further studies need to be conducted to elucidate the specific contribution of BER and oxidative DNA damage in different organ systems of experimental animals and humans, particularly during oxidative and carcinogenic challenge.

1.8 NNK and DNA repair systems

Studies on the effects of NNK on DNA repair systems are limited. To date, only 2 types of DNA repair have been shown to be affected by treatment with NNK; nucleotide excision repair (NER) and direct reversal repair. The NER pathway recognizes and removes bulky chemical adducts that can induce helix distortion in DNA. NER is also responsible for repairing cyclobutane pyrimidine dimers and photoproducts, two major kinds of lesions generated from UV light (de Laat et al., 1999). Using an in vitro NER assay, mice treated with 10 μmol of NNK i.p., had reduced activity for NER of POB adducted plasmid in extracts prepared from lung tissue (Brown and Massey, 2009). Interestingly, the same NNK treatment increased NER in liver extracts, suggesting inter-organ differences in susceptibility of NNK tumourigenicity.
Immunoblots showed an alteration in levels of key NER proteins, indicating that treatment with NNK can also alter protein levels of NER.

Direct reversal repair is the direct removal of a specific DNA adduct by a specialized class of proteins (Lindahl and Wood, 1999). For example, O^6^-methylguanine-DNA methyltransferase (MGMT) irreversibly binds and removes alkyl groups from O^6^-alkylguanine. *In vivo* treatment with NNK reduced MGMT activity in different lung cell types including type II cells and non-ciliated bronchiolar epithelial cells (Belinsky *et al*., 1988). In addition, MGMT activity was inhibited in cultured rat hepatocytes by OPB, a reactive intermediate generated by α-hydroxylation of NNK (Liu *et al*., 1992). The decline of MGMT activity could be due to direct adduct formation on the repair protein by reactive NNK metabolites (Mijal *et al*., 2004). Treatment with NNK could also induce alkyl or POB adducts in DNA which can act as competitive substrates during MGMT repair (Peterson *et al*., 2001). Some bulky POB adducts like O^6^-[4-oxo-4-(3-pyridyl)-butyl]guanine can compete with O^6^-alkylguanine for repair by MGMT, resulting in reduced removal of O^6^-alkyl guanine by MGMT (Wang *et al*., 1997; Peterson *et al*., 2001). These results demonstrate the possibility for NNK to alter the activity and levels of protein of DNA repair pathways in lung and liver.

With respect to BER, *in vivo* treatment with NNK has been studied in female OGG1 knockout mice (Igarashi *et al*., 2009). This study found NNK-treated knockout mice had significantly higher incidence and multiplicity of lung tumours compared to NNK-treated wild type mice. Antioxidants inhibited enhanced lung carcinogenesis in NNK-treated knockouts and NNK-treated wild type mice. These results suggest a potential role for an intact BER pathway in protecting against NNK-induced pulmonary carcinogenesis.
1.9 Research hypothesis and objectives

Lung cancer is the leading cause of cancer death worldwide. Although cigarettes are by far the largest cause of lung cancer cases among men and women, the mechanism by which cigarettes induce pulmonary carcinogenesis are not fully characterized. A potent pulmonary carcinogen found at significant levels in tobacco products is the tobacco specific nitrosamine, NNK. Treatment with NNK induced lung ACs in a variety of animal species. The metabolism of NNK by CYP enzymes results in the formation of reactive intermediates that can methylate and pyridyloxobutylate DNA and induce damage in major tumour suppressor genes and oncogenes. In addition, NNK has the potential to induce oxidative DNA damage. DNA repair systems are critical for protecting genomic integrity from chemical agents like NNK. Alterations in repair activities can result in persistent DNA lesions that can potentially exacerbate carcinogenesis. The overall objective of this research was to determine the ability of NNK to cause DNA oxidation in an established mouse model of NNK lung tumourigenicity, as well as to assess potential effects of NNK on BER activity in murine lung and liver. Treatment with NNK has been shown to alter NER and MGMT repair systems and may have a similar effect on BER.

**Hypothesis 1:** *In vivo* treatment with NNK induces oxidative DNA damage in murine lung and liver.

**Objective 1:** Determine the effect of *in vivo* treatment with NNK on levels of 8-OHdG in A/J mouse lung and liver.

**Hypothesis 2:** *In vivo* treatment with NNK alters BER activity in murine lung and liver.

**Objective 2:** Determine the effect of *in vivo* treatment with NNK on overall BER activity in A/J mouse lung and liver.
Chapter 2
MATERIALS AND METHODS

2.1 Reagents

To evaluate oxidative DNA damage, chemicals and reagents were obtained as follows:
NNK from Toronto Research Chemicals (North York, ON); 8-OHdG, 2’-deoxyguanosine (2’-dG), alkaline phosphatase, nuclease S1, nuclease P1 from Sigma (St. Louis MO); Ultrafree-MC 10,000 nominal molecular weight limit (NMWL) filter units from Millipore (Bedford, MA); Wako DNA Extractor TIS kit from Wako Chemicals USA, Inc. (Richmond, VA). To assess BER activity, chemicals and reagents were obtained as follows: proteinase K from Sigma (St. Louis, Mo); Slide-A-Lyzer dialysis cassettes 3500 molecular weight cutoff from Thermo Scientific (Rockford IL); EcoR1 from New England Biolabs (Beverley, MA); [α³²P] dGTP from Perkin Elmer (Waltham, MA). All other chemicals were reagent grade and were obtained from common commercial suppliers.

2.2 Animal Treatments

Female A/J mice, aged 7 to 8 weeks (Jackson Laboratories, Bar Harbour, ME) were housed with a 12 hour light/dark cycle and provided food and water ad libitum. Mice were treated with a single tumourigenic dose of 10 μmol NNK (~118 mg/kg NNK for mice that had an average weight of ~17 g) in saline (0.1 mL, i.p.) or 0.9% saline control (0.1 mL, i.p.). This NNK treatment protocol has been shown to induce 7 to 12 lung adenomas per A/J mouse 16 weeks later (Hecht et al., 1989). To avoid the use of anesthetics that could interfere with biochemical processes in lung or liver, mice were sacrificed by cervical dislocation 1, 2 or 24 hours following NNK administration. The time points were selected to assess BER activity and levels of oxidative DNA damage since NNK can induce substantial DNA damage in lung after the first
few hours after NNK administration (Peterson and Hecht, 1991; Chung and Xu, 1992; Tjaelve, 1991). Lungs and livers were perfused with TE buffer (10 mM Tris, 1 mM EDTA, pH 7.9) and excised. Organs were snap frozen in liquid nitrogen and stored at –80°C until nuclear extract preparation or DNA isolation. For each time point, 20 mice were treated with NNK and another 20 mice with saline. To determine BER activity, 4 sets of lungs were pooled together to produce 1 lung nuclear protein extract while 1 liver was used to produce 1 liver nuclear protein extract. To assess oxidative DNA damage, 1 set of lungs and 1 liver were required. A total of 120 mice were required for conducting 4 independent experiments at 1, 2 and 24 hour time points.

2.3 Isolation of DNA from lung and liver

DNA was isolated from lung and liver tissue using a Wako DNA TIS Extractor kit, according to the manufacturer’s recommendations with minor modifications: 0.1 mM deferoxamine mesylate (DFO) was added to the lysis solution, enzyme reaction solution, and TE buffer, while 0.3 mM DFO was added to the sodium iodide solution. DFO is an iron-chelating agent that prevents Fenton reactions and subsequent production of hydroxyl radicals that could artifactually oxidize DNA during sample preparation (Ravanat, 2005b). Briefly, lung and liver tissue (~0.15 g and ~0.6 g respectively) was pulverized into powder using a mortar and pestle in liquid nitrogen. Lysis solution was added to the tissue powder and homogenized to disrupt cellular membranes. The whole tissue lysate was centrifuged (600 x g for 10 minutes at 4°C) and RNAse, proteinase and an unspecified oxidation inhibitor were added and incubated at 37°C for 1 hour. The lysate was centrifuged (10,000 x g for 5 minutes at 4°C) and the supernatant kept. DNA was precipitated with 300 µL sodium iodide solution and washed with alcohol solutions. The resulting DNA was dried and dissolved in TE buffer containing 0.1 mM DFO. Concentration and purity of DNA were determined by spectrophotometry, using absorbance at
260 nm and 260 nm/280 nm absorbance ratio respectively. For purity of DNA, absorbance ratios between 1.8 and 2.0 were accepted. DNA was stored at –80°C until digestion steps.

2.4 DNA digestion

Thorough digestion of genomic DNA is necessary for accurate measurement of genomic 8-OHdG levels (Huang et al., 2001). Calf thymus DNA and pBluescript SK+ plasmid was used to optimize DNA digestion to ensure accuracy in the measurement of 8-OHdG levels. Nuclease P1 and S1 enzymes and incubation times were tested with calf thymus DNA, and selected based on which combinations produced the lowest 8-OHdG levels (data not shown). Preliminary results suggested that DNA digestion with nuclease P1 had the lowest levels of 8-OHdG. Incubating calf thymus DNA with nuclease P1 and alkaline phosphatase for different incubation times (ranging from total times of 15 minutes to 2 hours) did not seem to have an effect on lowering 8-OHdG levels. The final digestion method was performed using the method described by Lan et al. with minor modifications (Lan et al., 2000). Briefly, DNA was diluted with TE buffer containing 0.1 mM DFO to a final concentration of 0.15 mg/mL in 100 μL volume. Ten μL of 0.5 M sodium acetate (pH 5.1) and 1 μL of 1 M MgCl₂ were added to the DNA. To denature genomic DNA, samples were heated at 100°C for 5 minutes and immediately placed on ice for 5 minutes. One μL of nuclease P1 (4 mg/mL) was added to DNA and incubated at 37°C for 1 hour. Solution pH was adjusted to 7.8 by adding 1.25 μL of 1 M Tris (pH 10.5). Porcine alkaline phosphatase was prepared by dissolving in water with 0.1 mM DFO (0.1 U/μL). Ten μL of alkaline phosphatase was added to the sample and incubated at 37°C for 1 hour. Enzymes were precipitated by adding 2 μL of 5.8 M acetic acid. Samples were transferred to 10,000 NMWL filter units and centrifuged (12,000 x g for 20 minutes at 4°C), separating digested nucleosides from enzymes.
Samples were stored at –80°C until analysis. Gel electrophoresis was performed to check for comprehensive DNA digestion.

2.5 Determination of 8-OHdG and 2’-dG levels by high-performance liquid chromatography with electrochemical detection

To assess the levels of 8-OHdG and 2’-dG in genomic DNA, high-performance liquid chromatography with multichannel electrochemical detector (HPLC-ECD) (ESA Inc., Chelmsford, MA) was used. Compounds were separated on a Waters S-3 4.6 × 150 mm column with 0.1% methanol/99.9% 50 mM phosphate buffer (pH 5.5) at a flow rate of 1.0 mL/min (Bolin et al., 2004; Guindon et al., 2007). The four electrochemical detector channels were set to 0, 250, 475 and 875 mV. The ratio of 8-OHdG to 2’-dG was determined by comparing the concentration of each nucleoside in a digested DNA sample. The concentration of 8-OHdG and 2’-dG was quantified by determining the area under the curve (Figure 2.1) and interpolated to standard curves of 8-OHdG and 2’-dG. It should be noted that DNA isolation, digestion and HPLC analysis was carried out in one week, in order to prevent prolonged storage and artifactual oxidation (Kasai, 1997).

2.6 Cell-free whole tissue nuclear protein extract preparation

Cell-free whole tissue nuclear protein extracts were prepared from ~1.0 to 1.5 g of total tissue from livers of individual mice and from pooled lungs of 4 mice. These extracts, active in DNA repair synthesis, were prepared as described previously (Wood et al., 1988; Wood et al., 1995; Bedard et al., 2005). Extracts were stored at –80°C until repair reactions were carried out. Protein concentration of extracts were determined by the Lowry assay using bovine serum albumin as a standard (Lowry et al., 1951). Extracts from lung and liver typically contained ~5 and ~15 mg/mL protein, respectively.
Figure 2.1 Chromatograms demonstrating resolution of A) 2'-dG and B) 8-OHdG, isolated from lung DNA of mice following in vivo treatment with saline. Isocratic chromatography with electrochemical detection was performed using a Waters S-3 4.6 x 150 mm column and a mobile phase of 0.1% methanol/99.9% 50 mM phosphate buffer (v/v) (pH 5.5) at 1.0 mL/min at room temperature. The nucleoside 2'-dG was detected at 875 mV and 8-OHdG at 250mV. The area under the curve was calculated to determine the concentration of 2'-dG and 8-OHdG in samples. µA = microampere; nA = nanoampere; 2'-dG = 2'-deoxyguanosine; 8-OHdG = 8-hydroxydeoxyguanosine.
2.7 Preparation of 8-OHdG adducted plasmid

A 2961 bp plasmid derived from pBluescript SK+ (Stratagene, La Jolla, CA) was grown in *E. Coli* DH5α cells in 2xYT broth and isolated using a Qiagen Plasmid Mega Kit (Qiagen, Valencia, CA), as per manufacturer’s instructions with minor modifications. Instead of growing *E. Coli* in 500 mL liquid culture, a total of 1 L was grown in 2xYT liquid culture and eventually applied to a single anion-exchange column to isolate plasmid. The method to prepare DNA containing 8-OHdG adducts was adapted from Floyd *et al.* and Schneider *et al.* (Floyd *et al.*, 1989; Schneider *et al.*, 1990). The BER pathway can repair 8-OHdG adducts and its activity can be assessed using the *in vitro* DNA repair assay. Briefly, 50 μL of pBluescript SK+ plasmid (0.4 mg/mL in TE buffer) was placed on a parafilm-covered petri dish on ice. In the dark, 50 μL of methylene blue was added to the plasmid and exposed to white light from a 100 W tungsten lamp at a distance of 10 cm for 5 minutes. When irradiated with white light, methylene blue generates singlet oxygen that specifically reacts with guanine residues resulting in 8-OHdG adducts (Schneider *et al.*, 1990). A range of concentrations of methylene blue (final concentration of 1, 2, 5, 10 and 20 μM) was used to optimize the production of oxidized plasmid. Immediately after light exposure, irradiated DNA was precipitated with 20 μL of 5 M NaCl and 300 μL 100% ethanol. DNA was washed with 500 μL of 80% ethanol twice, dried down and re-dissolved in TE buffer. To determine the extent of 8-OHdG adduction, plasmid was thoroughly digested and subjected to HPLC-ECD. Gel electrophoresis was performed on a 1% agarose gel to determine extent of nicking damage in methylene blue damaged plasmid. DNA densitometry of the photograph of the UV-illuminated ethidium bromide gel (ChemImager 4000, Alpha Innotech Corporation, San Leandro, CA) was performed to quantify the percentage of nicked plasmid (~4 kb band) and intact supercoiled plasmid (~2 kb band) used in the *in vitro* repair assay.
2.8 *In vitro* base excision repair assay

The repair synthesis assay (Figure 2.2) was performed as described (Wood *et al.*, 1988; Wood *et al.*, 1995; Bedard *et al.*, 2005) with slight modifications. The 50 μL reaction mixture contained 400 ng of 8-OHdG damaged plasmid DNA or plasmid containing baseline DNA oxidation, 40 mM HEPES-KOH (pH 7.8), 0.5 mM dithiothreitol, 5.0 μM dGTP, 20 μM of each dATP, dCTP, and dTTP, 23 mM phosphocreatine, 18 μg bovine serum albumin (nuclease-free, Sigma Aldrich, St. Louis, MO), 2.5 μg creatine phosphokinase, 2.0 mM ATP, 5.0 mM MgCl₂, 0.4 mM EDTA, 100 mM KCl, 7.1 μCi [α³²P] dGTP, and 10 μg of lung or 25 μg of liver protein extract (concentrations listed are final concentrations). Untreated plasmid was used as a negative control and contained background levels of DNA oxidation. Samples were incubated for 3 hours at 30°C unless otherwise indicated. Reactions were terminated by adding EDTA (final concentration, 20 mM). Following incubation with 7.3 mg/mL RNase (final concentration, at 37°C for 10 minutes) and 925 μg/mL proteinase K with 0.5% SDS (final concentration, at 65°C for 30 minutes), plasmid DNA was purified by extraction with 250 μL of phenol/chloroform/isoamyl alcohol (25:24:1; v/v/v), precipitated with 1 mL of 100% ice-cold ethanol, washed in 1 mL of 80% ethanol, dried and linearized with 1 U of EcoR1. Gel electrophoresis was performed with the linearized plasmid on a 1% agarose gel. Plasmid DNA recovery was normalized by densitometry of the photograph of the UV-illuminated ethidium bromide gel (ChemImager 4000, Alpha Innotech Corporation, San Leandro, CA). The extent of [α³²P] dGTP incorporation was determined by phosphor imaging (Cyclone Plus Phosphor Imager, Perkin Elmer, Waltham, MA) of the dried gel (Figure 2.3). To determine damage-specific BER activity in lung and liver of saline control and NNK-treated mice, repair synthesis activity found in untreated plasmid containing baseline levels of oxidation was subtracted from repair synthesis...
Figure 2.2 Schematic of *in vitro* DNA repair synthesis assay on damaged plasmid DNA by nuclear extracts isolated from whole tissue. Squares on plasmid represent oxidative DNA lesions. 8-OHdG: 8-hydroxydeoxyguanosine; dGTP: deoxyguanosine triphosphate; EtBr: ethidium bromide (modified from Salles *et al.*, 1995).
Figure 2.3 UV-illuminated ethidium bromide stained agarose gel of linearized plasmid DNA and phosphor image demonstrating the ability of cell-free nuclear protein extracts to catalyze *in vitro* DNA repair synthesis. DNA in lanes 1 through 4 were incubated with nuclear protein from saline control lung. Lanes 1 and 2 are replicates and demonstrate background repair synthesis activity towards untreated plasmid DNA that contains background level of oxidation. Lanes 3 and 4 are replicates and show repair of 8-OHdG adducted plasmid.
activity found in plasmid containing oxidative DNA damage. The *in vitro* repair assay was performed in duplicate for each protein extract.

In addition, to optimize the *in vitro* repair assay, nuclear protein extracts from lungs and livers of control female A/J mice were prepared and tested. The level of oxidative damage in plasmid substrate, protein amount and incubation time were investigated to determine the linear range of each parameter in the *in vitro* repair assay. Each specific parameter of the BER assay was tested using separate aliquots from a single liver extract (prepared from 1 animal) or a single lung extract (prepared from 4 animals).

### 2.9 Data analysis

Statistically significant differences in levels of DNA oxidation and BER activity between NNK-treated and control groups were determined by unpaired Student’s *t*-test (GraphPad Prism 5 software). If heterogeneity of variance was present, data were transformed using the log-log function prior to statistical analysis. *P* < 0.05 was considered significant in all cases.
Chapter 3
RESULTS

3.1 Levels of 8-OHdG following *in vivo* treatment with NNK

The background levels of 8-OHdG in calf thymus DNA and freshly isolated pBluescript SK+ plasmid were on average 21.5 and 10.1 8-OHdG/10⁵ 2’-dG respectively. Gel electrophoresis revealed thorough digestion of calf thymus DNA and plasmid.

DNA oxidation was assessed in lungs of NNK-treated and saline control mice at each time point (Figure 3.1). No changes in 8-OHdG levels were observed between control mice (19.3 ± 3.9 8-OHdG/10⁵ 2’-dG) and NNK-treated mice (19.5 ± 3.3 8-OHdG/10⁵ 2’-dG) at the 1 hour time point (n=4, P = 0.928). At the 2 hour time point, there was an apparent 14% increase in 8-OHdG levels in the lungs of NNK-treated mice compared to control lungs; however, this was not statistically significant (n=4, P = 0.238). Lungs of control mice appeared to have a 15% increase in 8-OHdG levels compared to treated mice at 24 hours, but statistical significance was not attained (n=4, P = 0.119).

In liver, the levels of 8-OHdG were not statistically different between NNK-treated mice and control mice at any of the time points examined (Figure 3.2). An apparent 16% increase in 8-OHdG levels was observed in NNK-treated mice compared to controls at 1 hour, but was not statistically significant (n=4, P = 0.253). There appeared to be a 27% increase in 8-OHdG levels in NNK-treated mice compared to control mice at 2 hours; however this was not statistically significant (n=4, P = 0.166). At 24 hours, control mice (24.8 ± 6.7 8-OHdG/10⁵ 2’-dG) had similar levels of 8-OHdG compared to mice treated with NNK (23.9 ± 6.7 8-OHdG/10⁵ 2’-dG) and was not statistically significant (n=4, P = 0.864).
Figure 3.1 Effect of *in vivo* treatment with NNK on 8-OHdG levels in lung DNA. DNA was isolated from mice treated with 10 μmol NNK or saline and sacrificed 1 (A), 2 (B) or 24 (C) hr post-treatment. Results are presented as mean ± SD of 4 animals performed in triplicate and are expressed as the ratio of the concentration of 8-OHdG to 2’-dG. No statistically significant differences were found between NNK and saline control (*P > 0.05*, Student’s *t*-test).
Figure 3.2 Effect of *in vivo* treatment with NNK on 8-OHdG levels in liver DNA. DNA was isolated from mice treated with 10 μmol NNK or saline and sacrificed 1 (A), 2 (B) or 24 (C) hr post-treatment. Results are presented as mean ± SD of 4 animals performed in triplicate and are expressed as the ratio of the concentration of 8-OHdG to 2'-dG. No statistically significant differences were found between NNK and saline control (*P* > 0.05, Student’s *t*-test).
3.2 Characterization of 8-OHdG adducted plasmid DNA substrate for *in vitro* repair assay

Photoactivated methylene blue was used to induce 8-OHdG lesions in pBluescript SK+ plasmid (Figure 3.3 A). An increase in 8-OHdG levels was observed between untreated plasmid and plasmid treated with 1, 2, 5, 10 and 20 μM of methylene blue. Untreated plasmid contained a baseline level of oxidation of 16.1 ± 3.6 8-OHdG/10⁵ 2’-dG (n=4, mean ± SEM), while a maximum concentration of 20 μM methylene blue induced an average of 167 ± 16.4 8-OHdG/10⁵ 2’-dG (n=4, mean ± SEM). Plasmid was also assessed for nicking using gel electrophoresis and DNA densitometry (Figure 3.3 B). An increase in nicked plasmid was observed when increasing concentrations of methylene blue was used to induce oxidative DNA damage.

To choose an appropriate substrate for the *in vitro* repair assay, untreated plasmid and plasmid treated with 1, 2, 5, 10 and 20 μM of methylene blue were incubated with nuclear protein extracts prepared from control lung and liver to determine extent of repair activity (Figure 3.4). Oxidative DNA damage in the form of 8-OHdG ranged from ~16 8-OHdG/10⁵ 2’-dG lesions in untreated plasmid to ~170 8-OHdG/10⁵ 2’-dG in plasmid treated with 20 μM methylene blue.

BER was assessed by normalizing the amount of [α³²P] dGTP incorporated to plasmid recovery (μg). As anticipated, incubation of 10 μg of lung extract with untreated plasmid containing baseline oxidation resulted in the lowest levels of BER activity (Figure 3.4 A). Plasmid containing higher levels of 8-OHdG had increased levels of repair activity compared to untreated plasmid. A pronounced plateau in repair activity was observed in plasmid containing ~75 to ~175 8-OHdG/10⁵ 2’-dG. The incubation of 25 μg liver extract with untreated plasmid also produced the lowest levels of repair activity (Figure 3.4 B). An increase in repair activity was observed in plasmid containing up to ~75 8-OHdG/10⁵ 2’-dG and seemed to plateau at higher levels of oxidative DNA damage.
Figure 3.3 Characteristics of 8-OHdG adducted pBluescript SK+ plasmid by photo-oxidation of methylene blue. Plasmid was grown in and isolated from *E. coli* DH5α cells. Levels of 8-OHdG are presented as mean ± SEM of four independent experiments performed in duplicate and are expressed as the ratio of the concentration of 8-OHdG to 2'-dG (A). The percentage of nicked plasmid are expressed as mean ± SEM of three or four independent experiments (B).
Figure 3.4 Optimization of 8-OHdG adducted plasmid for *in vitro* BER assay. Protein extract from control lung (10 μg, A) or liver (25 μg, B) was incubated with plasmid containing varying amounts of 8-OHdG for 3 hr. Results are presented as mean of duplicates and are expressed as amol[^3]P]dGTP incorporated per μg of DNA. Plasmid treated with 5 μM methylene blue induced ~40 8-OHdG/10^5 2'-dG and was chosen to assess NNK’s effect on BER in lung and liver.
Plasmid treated with 5 µM methylene blue was selected to assess the effect of NNK on repair activity in both lung and liver. With this concentration of methylene blue, repair activity was ~5-fold higher compared to that in untreated plasmid in repair reactions containing lung or liver extract. This plasmid substrate contained ~40 8-OHdG/10^5 2’-dG (Figure 3.3) and was used in subsequent in vitro repair assays.

3.3 Optimization of nuclear protein extract amount for in vitro repair assay

Saline control lung and liver extract was tested with the in vitro repair assay to select an optimal protein amount to assess BER activity. A range of concentrations of both nuclear protein extracts were incubated with plasmid treated with 5 µM methylene blue for 3 hours. Repair reactions containing neither lung nor liver extracts resulted in no incorporation of [α^32P] dGTP and thus no BER activity. Incubation reactions containing a maximum of 25 µg of lung extract resulted in an increase in repair activity (Figure 3.5 A). BER activity reached a plateau in reactions incubated with higher amounts of lung extract. Similarly, repair reactions containing a maximum of 50 µg of liver extract appeared to increase BER activity in a linear fashion (Figure 3.5 B). Higher amounts of liver extract resulted in a plateau of BER activity. Ten µg of lung extract and 25 µg of liver extract were chosen to assess BER activity in organs of control and NNK-treated mice as increases and decreases in BER activity were detectable with these amounts of protein.

3.4 Optimization of incubation time for in vitro repair assay

To select an optimal incubation time for the repair assay, repair activity was assessed over time with control lung and liver nuclear protein extracts. Repair reactions contained plasmid oxidized with 5 µM methylene blue and 10 µg of lung extract or 25 µg of liver extract. An
Figure 3.5 Optimization of protein amount for *in vitro* BER assay. Varying amounts of protein extract from control lung (A) or liver (B) were incubated with plasmid damaged by 5 μM methylene blue for 3 hr. This parameter was tested using a single lung extract (A) or separate aliquots from a single liver extract (B). Results are presented as mean of duplicates (A) or mean ± SEM of three independent experiments performed in duplicate (B). Ten μg of lung protein and 25 μg of liver protein was chosen to assess the effect of NNK on BER.
increase in repair activity was observed in repair reactions containing 10 µg of lung extract or 25 µg of liver extract across all incubation times (Figure 3.6 A and B). The levels of BER activity appeared to be linear at a maximum incubation time of 4 hours in both lung and liver. An incubation time of 3 hours was picked to assess BER in lung and liver of control and NNK-treated mice.

3.5 Effect of in vivo treatment of mice with NNK on BER in lung and liver

Damage-specific BER activity was assessed in lung and liver of mice following in vivo treatment with NNK using the optimized parameters. No statistically significant differences were observed between lung extracts prepared from NNK-treated mice and control mice at each time point (Figure 3.7). At the 1 hour time point, mice treated with NNK appeared to have a 34% reduction in BER activity in lung tissue compared to controls, however statistical significance was not attained (n=4, P = 0.114). An apparent 13% decrease in BER activity was observed in lungs of NNK-treated mice at 2 hours, but was not statistically significant (n=4, P = 0.535). At 24 hours lungs of NNK-treated mice appeared to have a 21% decline in repair activity, but was not statistically significant (n=4, P = 0.146).

In liver, no statistically significant differences were observed at the 1 hour time point as repair activities of NNK treatment and control groups were similar (n=4, P = 0.818) (Figure 3.8). At 2 hours NNK-treated mice had an apparent 16% reduction in BER activity in liver; however this was not statistically significant (n=3 or 4, P = 0.163). BER activity was slightly reduced in livers of NNK-treated mice at the 24 hour time point but statistical significance was not attained (n=4, P = 0.675).
Figure 3.6 Optimization of incubation time for *in vitro* BER assay. Protein extracts from control lung (10 μg, A) or liver (25 μg, B) were incubated for varying amounts of time with plasmid damaged by 5 μM methylene blue. This parameter was tested using a single lung extract (A) or separate aliquots from a single liver extract (B). Results are presented as mean of duplicates (A) or mean ± SEM of three independent experiments performed in duplicate (B). A 3 hr incubation time was chosen to assess NNK’s effect on BER in lung and liver.
Figure 3.7 Effect of *in vivo* treatment with NNK on *in vitro* BER in mouse lung extracts towards 8-OHdG adducted plasmid. Extracts were prepared from mice treated with 10 μmol NNK or saline and sacrificed 1 (A), 2 (B) or 24 (C) hr post-treatment. Results are presented as mean ± SD and expressed as damage-specific BER. No statistically significant differences were found between NNK and saline control at each time point (*P > 0.05*, Student’s *t*-test).
Figure 3.8 Effect of in vivo treatment with NNK on in vitro BER in mouse liver extracts towards 8-OHdG adducted plasmid. Extracts were prepared from mice treated with 10 μmol NNK or saline and sacrificed 1 (A), 2 (B) or 24 (C) hr post-treatment. Results are presented as mean ± SD and expressed as damage-specific BER. No statistically significant differences were found between NNK and saline control at each time point (P > 0.05, Student’s t-test).
Chapter 4
GENERAL DISCUSSION

In the present study, female A/J mice were treated with a tumourigenic dose of NNK and the level of DNA oxidation and BER activity was determined in lung and liver of control and NNK-treated mice.

4.1 *In vivo* treatment with NNK does not induce oxidative DNA damage

NNK-induced carcinogenesis typically involves metabolic activation to generate reactive intermediates that methylate or pyridyloxobutylate DNA. *In vivo* treatment with NNK in rodents, induces DNA damage in oncogenes and tumour suppressor genes that play a critical role in carcinogenesis (Hecht, 1999). In addition, *in vivo* treatment with NNK has been reported to induce oxidative DNA damage in the form of 8-OHdG in tissues such as lung and liver in mice (Chung and Xu, 1992; Rosa *et al*., 1998; Weitberg and Corvese, 1993). The consequences of excess 8-OHdG, a major oxidative DNA lesion, include G→T transversion mutations in oncogenes and tumour suppressor genes that could contribute to carcinogenesis (Klaunig and Kamendulis, 2004).

Reported basal levels of DNA oxidation in biological systems have been inconsistent, with values of 8-OHdG differing by ~2 orders of magnitude (Collins *et al*., 2004; Ravanat, 2005a). The large variation in 8-OHdG levels can largely be attributed to different methods and techniques used to isolate and assess DNA oxidation. For example, the levels of 8-OHdG in pig liver DNA ranged from 0.21 to 44.1 8-OHdG per 10^5 2’-dG using HPLC-ECD and gas chromatography-mass spectrometry (GC-MS) (Collins *et al*., 2004). Guanine is readily oxidizable during sample preparation and some techniques are conducive to spurious oxidation that can overestimate the levels of DNA oxidation. GC-MS is prone to high levels of artifactual
oxidation during derivatization which require high temperature and a silylating agent to make DNA samples volatile for separation (Cadet et al., 1998). Conversely, the lowest levels of basal 8-OHdG have been observed when using enzymes specific for 8-OHdG (Ravanat, 2005a). In enzymatic nicking assays such as the comet assay, levels of 8-OHdG can range from < 1 to 10 8-OHdG residues per 10^6 2’-dG. It is unclear whether enzymes bind all 8-OHdG sites in genomic DNA. Failure to do so would underestimate the levels of 8-OHdG (Halliwell and Whiteman, 2004; Ravanat, 2005a). When measuring background levels of oxidative DNA damage in control tissues and cells, HPLC-ECD has generated values as low as several 8-OHdG per 10^6 2’-dG in human lymphocytes and as high as 20 8-OHdG per 10^5 2’-dG in untreated murine liver (Zhang et al., 2004; ESCODD et al., 2005; Guindon et al., 2007).

In the present study, DNA of lung and liver was isolated, digested and assessed for the ratio of 8-OHdG per 10^5 2’-dG using HPLC-ECD. For chromatographic methods including HPLC-ECD, the addition of metal chelators and antioxidants during sample preparation is standard practice to reduce spurious oxidation (European Standards Committee on Oxidative DNA Damage, 2003; Ravanat, 2005a). DFO, an iron chelator that prevents spurious oxidation via Fenton reactions, and an additional unspecified antioxidant (included in the Wako DNA Extractor TIS kit) were used when isolating DNA. In addition, a sodium iodide extraction method was utilized to avoid the use of phenol and chloroform extraction which can artificially enhance 8-OHdG levels (Claycamp, 1992; Ravanat, 2005a). DNA digestion was also optimized for thorough digestion to reduce spurious oxidation (Huang et al., 2001). Gel electrophoresis of the DNA digests revealed absence of high molecular weight DNA, indicating that genomic DNA was thoroughly digested. DNA isolation, digestion and HPLC analysis of control and NNK-treated samples were carried within one week to minimize artifactual DNA oxidation. Samples
from control and NNK-treated animals for a given time point were processed at the same time to minimize the potential impact of inter-day assay variability (Kasai, 1997).

The levels of 8-OHdG were comparable to a number of studies found in the literature. The background levels of 8-OHdG in calf thymus DNA were 21.5 8-OHdG per $10^5$ 2'-dG and were comparable to optimal levels determined by the European Standards Committee on Oxidative DNA Damage which ranged from 1.9 to 21.3 8-OHdG per $10^5$ 2'-dG when using HPLC-ECD (Lunec, 1998). These results suggest that artifactual oxidation was minimal when using the current protocol in the present study. In addition, control levels of oxidative DNA damage in murine lung and liver were not variable at each time point, and comparable to other studies that assessed 8-OHdG in such tissues using HPLC-ECD (Zhang et al., 2004; Guindon et al., 2007).

In the present study, 8-OHdG was assayed in lungs and livers of mice treated with a tumourigenic dose (10 μmol) of NNK after 1, 2 and 24 hours. No statistically significant effects in 8-OHdG levels were observed in lung or liver of mice treated with NNK at each time point, indicating in vivo treatment with NNK did not induce oxidative DNA damage. However, a trend towards elevated 8-OHdG levels was observed in liver and less so in lungs from NNK-treated mice in the first 2 hours. It is important to recognize that these trends were non-significant, as an apparent 27% increase in 8-OHdG levels in liver (n=4, $P = 0.166$) and apparent 14% increase in 8-OHdG levels in lung (n=4, $P = 0.238$) was observed at 2 hours. Although not statistically significant this trend towards elevated 8-OHdG levels is similar to other studies that reported an increase in 8-OHdG levels in lung and liver 2 hours after NNK administration, with a decrease to basal levels after 24 hours (Chung and Xu, 1992; Xu et al., 1992; Rosa et al., 1998).
Although some studies have reported oxidative DNA damage in experimental animals after NNK treatment, this is not always the case. In some studies, *in vivo* treatment with NNK failed to cause statistically significant changes in DNA oxidation. Treatment with 19.3 µmol of NNK i.p. appeared to increase the levels of 8-OHdG in A/J mouse lung and liver 2 hours after administration, but the effect was not statistically significant (Chung and Xu, 1992). Furthermore, treatment of A/J mice with multiple doses of NNK (11.65 mg/kg body weight, via gavage, 3 times weekly for 10 weeks) did not significantly affect 8-OHdG levels in liver 2 hours post-treatment (Xu et al., 1992). In such studies, NNK could have altered oxidative DNA damage in specific cell types of lung and liver but these effects may not have been detected when analyzing oxidative DNA damage from whole lung and liver. Similar trends were observed in the current study, with statistically non-significant increases in 8-OHdG levels in lung and liver following NNK treatment, suggesting NNK may actually have limited effect on DNA oxidation status.

### 4.2 *In vivo* treatment with NNK does not alter overall BER activity

DNA repair systems are critical for protecting and maintaining the genome from exogenous and endogenous damaging agents. Several DNA repair systems have been identified and categorized based on the type of damage repaired. A major function of the BER pathway is to identify and remove bases that have been damaged by ROS. The effects of NNK have previously been studied on nucleotide excision repair (NER) and direct reversal repair systems, but have not on BER.

In the present study, an *in vitro* repair assay was optimized to assess BER in lung and liver of mice treated with NNK. To assess BER activity, a plasmid substrate containing 8-OHdG lesions was incubated with cell-free nuclear protein extracts prepared from murine lung and liver.
Methylene blue irradiated with white light was used to induce oxidative DNA damage in the plasmid substrate. During photo-oxidation, methylene blue is activated into a transitory excited state which in turn can react with stable oxygen to generate reactive singlet oxygen (Floyd et al., 1989). When exposed to DNA, singlet oxygen has high specificity for guanine residues and usually generates 8-OHdG lesions (Epe, 1991). An increase in 8-OHdG levels was observed when using a range of methylene blue concentrations to induce oxidative damage. The levels of 8-OHdG were dependent on the final concentration of methylene blue during exposure to light. These results are consistent with literature that showed increasing 8-OHdG levels in DNA treated with increasing concentrations of methylene blue irradiated with light (Floyd et al., 1989; Schneider et al., 1990).

Plasmid was also assessed for nicking status using gel electrophoresis and DNA densitometry. Methylene blue was selected as an oxidizing agent as it preferentially induces 8-OHdG over DNA strand breaks that could potentially be repaired by systems other than BER (Schneider et al., 1990). In the current study, only 3% of the untreated plasmid was nicked, leaving 97% in supercoiled form. Plasmid treated with 5 μM methylene blue and subsequently chosen to assess BER in lung and liver was 12% nicked, leaving 88% in supercoiled form. Although a small percentage of both types of plasmid were nicked, DNA single strand nicks have been shown to stimulate very little incorporation of radiolabelled nucleotides when using a similar in vitro repair assay that assessed NER (Wood et al., 1988; Brown et al., 2007). Similarly, no significant differences were observed in NER towards nicked and supercoiled plasmid that contained POB adducts, indicating no artifactual increase in damage-specific repair activity due to nicks (Brown et al., 2007). These findings suggest that the limited amounts of nicked DNA present in the plasmid substrate and used in the current study likely had minimal impact on the BER activities measured.
When choosing an appropriate plasmid substrate to assess BER, several plasmid substrates were prepared with varying levels of DNA oxidation. BER activity was dependent on the amount of 8-OHdG induced by methylene blue within the plasmid substrate. Untreated plasmid containing background levels of 8-OHdG had the lowest activity compared to plasmid substrates treated with methylene blue. An upper limit of BER activity was reached in plasmid substrate treated with high concentrations of methylene blue that induced high levels of 8-OHdG. The repair system provided by the lung and liver extract could have been limiting, resulting in a plateau in repair activity. Plasmid treated with 5 μM methylene blue induced ~40 8-OHdG per $10^5$ 2'-dG and was selected for the assay since repair in this plasmid was ~5-fold higher compared to untreated plasmid.

BER activity was also protein concentration-dependent and repair duration-dependent. When assessing protein concentration in the assay, nuclear extract was required for repair activity as repair reactions that contained no protein had no incorporation of [$\alpha^{32}$P] dGTP. A plateau in BER activity was observed when incubations contained > 30 μg of lung extract or > 50 μg of liver extract, presumably due to limiting amounts of damaged plasmid substrate. It should be noted that at higher protein amounts (ranging from 100 to 200 μg of liver extract in particular), a decrease in repair activity was sometimes observed (data not shown). The presence of excessive amount of proteins may have interfered with recognition and binding of 8-OHdG lesions, reducing overall efficiency and repair activity. When assessing incubation time, an apparent linear increase in repair activity was observed in murine lung and liver over 4 hours. Repair reactions that were terminated immediately had some repair activity. BER proteins may begin to initiate repair processes upon addition of oxidized plasmid substrate followed by reaction termination. These results were similar to studies that demonstrated BER activity in extracts from rat brain was dependent on protein concentration and incubation time (Lan et al., 2003;
Chen et al., 2000). In those studies, an upper limit in BER activity was also observed at protein amounts > 30 μg and incubation times > 8 hours.

When assessing the effects of NNK on BER in liver and lung, selection of an oxidized plasmid substrate, protein concentration and incubation time was chosen based on the ability to detect increases and decreases in BER activity. Plasmid treated with 5 μM methylene blue, a 3 hour incubation time and 10 μg of lung or 25 μg of liver extract were chosen for the present in vitro repair assay. No statistically significant differences in BER were observed in lung or liver between control and NNK-treated mice at 1, 2 or 24 hours. Hence, administration of a tumourigenic dose of NNK did not affect BER activity up to 24 hours post treatment. In previous studies, NNK was found to inhibit NER and O\textsuperscript{6}-methylguanine-DNA methyltransferase (MGMT) in lung, and increase NER in liver (Belinsky et al., 1988; Liu et al., 1992; Brown and Massey, 2009) but failed to alter BER activity in the current study. As a result, it appears that NNK could selectively modify DNA repair pathways like NER and MGMT, while sparing others like BER. However, it is also possible that the previously reported effectiveness of NNK at altering non-BER DNA repair systems could be due to differences in: doses of NNK; experimental designs; species and strains of animals; and time points. For example, MGMT inhibition by NNK was demonstrated in specific lung cell types of rats treated with multiple doses of NNK (Belinsky et al., 1988). Furthermore, rat hepatocytes treated with OPB, a reactive metabolite of NNK, had inhibited MGMT activity (Liu et al., 1992). Nonetheless, NNK’s inhibition of NER in lung was studied using the same dose and strain of mouse and a time course similar to that of the current BER study (Brown and Massey, 2009).

One mechanism by which NNK could modify specific repair pathways is by forming specific protein adducts. Treatment with NNK has been shown to pyridyloxobutylate proteins
including hemoglobin and MGMT (Murphy et al., 1990; Mijal et al., 2004). Electrophilic intermediates generated during NNK metabolism may preferentially bind to major proteins of NER or MGMT systems modifying their overall repair activity, but may react less with BER proteins, perhaps due to lower affinity or lack of an available specific amino acid binding motif.

It should be noted that an apparent decreasing trend in BER (by 34% and 21%, at 1 and 24 hours post administration respectively) was observed in lungs of NNK-treated mice, although it was not statistically significant. A potential explanation for the lack of significant effect of NNK on repair of the oxidized plasmid is repair of 8-OHdG by additional mechanisms. Treatment with NNK could have inhibited BER activity in lung, but this inhibition could have been mitigated by other functional BER glycosylases or non-BER DNA repair mechanisms present in the nuclear extracts. Humans and mice both have several BER glycosylases that have overlapping function and redundancy to ensure extra protection against frequently occurring oxidative DNA lesions, like 8-OHdG (Hazra et al., 2003; Cooke et al., 2003). For example, BER glycosylases such as OGG1 and Nei-like 1 both repair 8-OHdG adducts in DNA and either glycosylase could act as a backup in case one has compromised activity (Hazra et al., 2003). In some cases mismatch repair and NER systems have also been shown to repair 8-OHdG lesions in DNA, and could alleviate the effect of decreased BER (Reardon et al., 1997; Colussi et al., 2002; Patel et al., 2007). In the present study, these additional repair mechanisms could have also played a role in reducing the effect of NNK on BER and protecting the genome from oxidative DNA in lungs of mice.

A tumourigenic dose of NNK (10 µmol) given i.p. also had no significant effect on BER activity in liver. Interestingly, the same dose and route of administration of NNK increased NER activity and levels of specific NER proteins in liver of A/J mice 24 hours post administration (Brown and Massey, 2009). Hence, NNK increases NER activity in liver while having no effect
on BER. These results are consistent with the fact that NER and BER are regulated differently. For example, post-translational modifications such as phosphorylation, ubiquitylation and sumoylation occur to varying degrees to proteins in the NER and BER pathways and could affect enzyme recruitment, DNA-protein interactions, protein-protein interactions and catalytic function of each DNA repair system (Huang and D'Andrea, 2006; Branzei and Foiani, 2008).

4.3 Conclusions

The research presented in this thesis has demonstrated that a single in vivo administration of NNK did not induce oxidative DNA damage in A/J mouse lung or liver at 1, 2 or 24 hours. Only a slight increasing trend in oxidative DNA damage was observed in lung and liver that was not statistically significant. Additionally, this was the first study to test the effects of NNK on BER. Using an optimized in vitro repair assay, NNK did not alter overall BER activity in lung and liver at each time point. A decreasing trend in BER activity was observed in lung that was not statistically significant.

4.4 Future directions

Although not statistically significant, an increasing trend in 8-OHdG and decreasing trend in BER activity in lung and liver of mice treated with NNK was observed and should be further investigated. This was the first study to test the effect of NNK on BER and supplementary studies should be carried out to verify the present findings. Additional experiments could be conducted to determine if NNK has effect on BER and DNA oxidation over a daily, weekly or monthly time course. The current research investigated potential effects of NNK in lung and liver acutely at 1, 2 and 24 hours post administration. The dose of NNK employed in the current study (10 µmol) has been shown to cause induction of lung adenomas in mice 16 weeks later (Hecht et al., 1989). The progression of lung adenomas to malignant adenocarcinomas was observed in
lungs 50 weeks post NNK administration (Hecht, 1998). Assessing NNK’s effect on DNA oxidation and BER in lung and liver over a longer period of time could further elucidate its role during carcinogenesis. Furthermore, the current research used a single i.p. dose of NNK that does not accurately model chronic smoking behaviour in humans. While a single 10 µmol dose given i.p. will most likely induce pulmonary carcinogenesis in mice, chronic administration of NNK at lower doses over several weeks more closely resembles smoking behaviour.

Investigating the effects of NNK is important for understanding tobacco-induced carcinogenesis. Substantial experimental data has shown NNK to be one of the most potent pulmonary carcinogens found in cigarettes and tobacco products and most likely plays a role in tobacco-induced human cancers. Studying NNK in isolation from a chemical agent as complex as tobacco smoke is helpful in specifically characterizing the molecular mechanism by which a major component of cigarettes affects DNA repair processes. However, to expand the scope of this project, a second approach should also be taken that investigates the effect of cigarette smoke on repair activity in animal models. NNK is only one component of cigarettes and tobacco smoke that contain over 4000 chemicals including 55 known carcinogens. The current study is limited by only investigating the effects of NNK on DNA damage and repair. Exposing experimental animals to cigarette smoke would represent human smoking more accurately by having inhalation as the primary route of administration as well as treating with agents that have the same chemical composition as tobacco smoke. BER and other repair processes could be assessed in lung and liver in these studies.

If statistically significant differences in BER activity were observed in future studies, the next logical step would be to determine where and how changes were occurring in the pathway. A modified in vitro oligonucleotide repair assay would be carried out to ascertain if changes occurred during the recognition and excision step, or nucleotide insertion and ligation step of the
BER pathway. If changes in activity occurred at both or either steps, immunoblots of key BER proteins would be performed on control and NNK-treated animals to determine if changes in repair activity were due to variation in protein levels. Real-time reverse transcription polymerase chain reaction could be performed to determine if potential changes in levels of BER proteins were due to changes in mRNA transcript levels. A nuclear run-on assay could also be used to determine if NNK alters transcription rates of particular BER genes. Reactive intermediates generated by metabolism of NNK have been shown to adduct proteins such as hemoglobin (Murphy et al., 1990) and could methylate or pyridyloxobutylate key BER proteins that could change protein activity or stability. Adducted BER proteins could be separated using SDS-PAGE and following immunoblotting, excised, digested and subjected to mass spectrometry for identification.

Potential changes in the activity of BER proteins could also be due to changes in post-translational modifications. Phosphorylation, sumoylation and ubiquitylation of repair proteins are important for regulating several types of DNA repair (Branzei and Foiani, 2008; Huang and D’Andrea, 2006). For example, sumoylation of certain BER glycosylases can alter catalytic activity for specific substrates, as well as reduce affinity for the AP site which allows access to DNA for further processing by additional BER proteins (Hardeland et al., 2002; Steinacher and Schär, 2005). Enzymatic techniques and mass spectrometric analysis could be used to determine if NNK-induced changes in BER activity were due to alterations in post-translational modifications on specific proteins. These types of experiments would ultimately investigate components of NNK’s mechanism of action and could be helpful in identifying susceptible individuals and future development of novel preventative therapies or treatments for tobacco-induced cancer.
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