BIOTRANSFORMATION AND DNA REPAIR IN 4-(METHYLNITROSAMINO)-1-(3-PYRIDYL)-1-BUTANONE-INDUCED PULMONARY CARCINOGENESIS

by

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ABSTRACT

Pamela J Brown: Biotransformation and DNA repair in 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone-induced Pulmonary Carcinogenesis. Ph.D. Thesis, Queen's University at Kingston, November 2008.

Studies described in this thesis were at aimed at characterizing the mechanisms involved in the pulmonary carcinogenicity of the tobacco-specific nitrosamine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), by addressing two critical determinants of carcinogenicity; biotransformation and DNA repair.

The contributions of cytochrome P450 (CYP) 2A13 and CYP2A6 to NNK biotransformation in human lung microsomes were investigated. Based on total bioactivation and detoxification of NNK and its keto-reduced metabolite, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), subjects could be classified as either high or low bioactivators and detoxifiers. Data from all of 29 individuals revealed no correlations between levels of CYP2A mRNA, enzyme activity or immunoinhibition and the degree of total NNK bioactivation or detoxification. However, subgroups were identified for whom CYP2A13 mRNA correlated with total NNK and NNAL bioactivation (n=4) and NNAL detoxification (n=5). Although results do not support CYP2A13 or CYP2A6 as predominant contributors to NNK metabolism in lung of all individuals, CYP2A13 appears to be important in some.

The involvement of nucleotide excision repair (NER) in the repair of NNK-induced DNA pyridyloxobutylation was assessed. Extracts from NER-deficient cells

were less active at repairing pyridyloxobutyl (POB) adducts on plasmid DNA than were extracts from normal cells, and NER-deficient cells were more susceptible to 4-(acetoxymethylnitrosamino)-1-(3-pyridyl)-1-butanone (NNKOAc)-induced cytotoxicity, demonstrating the participation of NER in the repair of POB-DNA adducts.

The role of DNA repair in contributing to inter-organ susceptibility to NNK-induced carcinogenesis was investigated. POB adduct repair was greater in extracts from mouse liver than lung, and activities in lungs of NNK-treated mice were lower than those of saline-treated mice, while repair was 3 times higher in livers of NNK-treated mice relative to control. NNK treatment decreased incision of POB adducts by 92 % in lung extracts and increased incision by 169 % in liver extracts. In addition, NNK altered the levels and binding to POB damage of key incision proteins. These results suggest that lower NER incision activity and NNK-mediated alterations in levels and activities of incision proteins contribute to the relative susceptibility of mouse lung to NNK-induced carcinogenesis.

Keywords: biotransformation, cytochromes P450, DNA repair, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, nucleotide excision repair, pulmonary carcinogenesis

CO-AUTHORSHIP

This research was conducted by the candidate Pamela J Brown, under the supervision of Dr. Thomas E Massey with the following co-authorships.

Chapter 2: Co-authored by Dr. Leanne L Bedard, Dr. Ken R Reid and Dr. Dimitri Petsikas.

Chapter 3: Co-authored by Dr. Leanne L Bedard.

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LIST OF ABBREVIATIONS

A	adenine
AC	adenocarcinoma
AGT	O^6-alkylguanine-DNA alkyltransferases
amol	atto (10^{-18}) moles
AP	apurinic/apyrimidinic
Arg	arginine
ASA	acetylsalicylic acid
ATP	adenosine triphosphate
BER	base excision repair
bp	base pair
BSA	bovine serum albumin
C	cytosine
cDNA	complementary deoxyribonucleic acid
CO	carbon monoxide
CSA	Cockayne's syndrome protein A
CSB	Cockayne's syndrome protein B
CYP	cytochrome P450
CYP2A13	cytochrome P450 2A13
CYP2A13	cytochrome P450 2A13 gene
CYP2A6	cytochrome P450 2A6

CYP2A6	cytochrome P450 2A6 gene
Cys	cysteine
DDB	damaged DNA binding protein
diol	4-hydroxy-1-(3-pyridyl)-1-butanol
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DNA pol	DNA polymerase
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
dTTP	deoxythymidine triphosphate
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ERCC1	excision repair cross-complementing group 1
EtBr	ethidium bromide
F (tables)	female
G	guanine
GAPDH	glyceraldehydes 3-phosphate dehydrogenase
GGR	global genome repair
^{3}H	tritium
HA	hydroxy acid
HEPES	*N*-2-hydroxy-ethylpiperazine-*N*-2-ethanesulfonic acid

[5-^{3}H]NNK	NNK tritiated at position 5 of the pyridine ring
HPB	4-hydoxy-1-(3-pyridyl)-1-butanone
HPLC	high performance liquid chromatography
hydroxy acid	4-hydroxy-4-(3-pyridyl) butyric acid
11β-HSD1	11β-hydroxysteroid dehydrogenase type 1
in vitro	outside the living body
in vivo	inside the living body
ip	intraperitoneal
iso-NNAC	4-(methylnitrosamino)-4-(3-pyridyl)butyric acid
iso-NNAL	4-(methylnitrosamino)-4-(3-pyridyl)-1-butanol
KA	keto acid
KAL	keto alcohol
keto acid	4-oxo-4-(3-pyridyl) butyric acid
keto alcohol	4-oxo-4-(3-pyridyl)-1-butanol
K-*ras*	Kirsten-*ras*
LCC	large cell carcinoma
LOH	loss of heterozygosity
LOX	lipoxygenase
M (tables)	male
7-mG	7-methylguanine
mRNA	messenger ribonucleic acid
N/A	not available

NAB	*N*-nitrosoanabasine
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NAT	*N*-nitrosoanatabine
NER	nucleotide excision repair
NNA	nicotine-derived nitrosaminoaldehyde
NNAL	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol
NNALOAc	4-(acetoxymethylnitrosamino)-(3-pyridyl)-1-butanol
NNAL-*O*-Glu	β-*O*-[4-(methylnitrosamino)-1-(3-pyridyl)-1-but-1-yl]-D-glucosiduronic acid
NNAL-*N*-Glu	β-*N*-[4-(methylnitrosamino)-1-(3-pyridyl)-1-but-1-yl]-D-glucosiduronic acid
NNAL-*N*-oxide	4-(methylnitrosamino)-1-(3-pyridyl *N*-oxide)-1-butanol
NNK	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
NNK-N-oxide	4-(methylnitrosamino)-1-(3-pyridyl *N*-oxide)-1-butanone
NNKOAc	4-(acetoxymethylnitrosamino)-1-(3-pyridyl)-1-butanone
NNN	*N*-nitrosonornicotine
NSAID	non-steroidal anti-inflammatroy drug
NSCLC	non-small cell lung carcinoma
NTH	neutral thermal hydrolysis
O^6-mG	O^6-methylguanine
O^2-POB-Cyt	O^2-[4-(3-pyridyl)-4-oxobut-1-yl]cytosine

O^2-POB-dCyd	O^2-[4-(3-pyridyl)-4-oxobut-1-yl]-2'-deoxycytidine
O^6-POB-dGuo	O^6-[4-(3-pyridyl)-4-oxobut-1-yl]-2'-deoxyguanosine
O^2-POB-dThd	O^2-[4-(3-pyridyl)-4-oxobut-1-yl]thymidine
O^4-mT	O^4-methylthymine
Pckyrs	pack years
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PHB	pyridylhydroxybutyl
PHS	prostaglandin H synthase
POB	pyridyloxobutyl
7-POB-dGuo	7-[4-(3-pyridyl)-4-oxobut-1-yl]-2'-deoxyguanosine
7-POB-Gua	7-[4-(3-pyridyl)-4-oxobut-1-yl]guanine
pyridyloxobutyl	4-(3-pyridyl)-4-oxobut-1-yl
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RNAPII	RNA polymerase II
RT-PCR	reverse transcriptase polymerase chain reaction
RPA	replication protein A
SCC	squamous cell carcinoma
SCLC	small-cell lung cancer
SDS	sodium dodecyl sulfate
SPE	solid phase extraction

T	thymine
TCR	transcription coupled repair
TFIIH	transcription factor II H
TSNA	tobacco-specific nitrosamine
U	enzymatic units
UGT	UDP-glucuronosyltransferase
UV	ultraviolet
XP	xeroderma pigmentosum
XPA	xeroderma pigmentosum group A
XPB	xeroderma pigmentosum group B
XPC	xeroderma pigmentosum group C
XPD	xeroderma pigmentosum group D
XPF	xeroderma pigmentosum group F
XPG	xeroderma pigmentosum group G

CHAPTER 1

GENERAL INTRODUCTION

1.1 STATEMENT OF RESEARCH PROBLEM

Lung cancer is the leading cause of cancer-related death in the world and cigarette smoking is recognized as the most important contributing factor (Hecht, 2003). Since approximately 90% of all lung cancer cases are attributable to smoking, it is likely that if tobacco use were to cease, lung cancer incidence would decline dramatically. Although there has been heightened public awareness of the hazards attributed to the use of tobacco, statistics on tobacco use are still daunting. Worldwide, approximately 10 million cigarettes are purchased every minute, 15 billion are sold each day, and about 5 trillion are produced and used annually (World Health Organization, 2008). As well, it does not appear that tobacco use will decline in the years ahead as youth are still beginning to smoke at a rate of 80,000 to 100,000 people each day worldwide and it is estimated that one in five teens aged 13 to 15 smoke cigarettes (World Health Organization, 2008). The predicted high incidence of lung cancer in future years and the poor prognosis and high lethality related to this disease continues to provide incentive to elucidate the molecular mechanisms involved in lung cancer induction. Thus, further understanding and characterization of these processes may lead to the development of new therapeutic strategies aimed at the prevention and/or treatment of lung cancer.

The process of cancer induction involves a series of interdependent steps (Figure 1.1). Most carcinogens exist as procarcinogens and require conversion into reactive

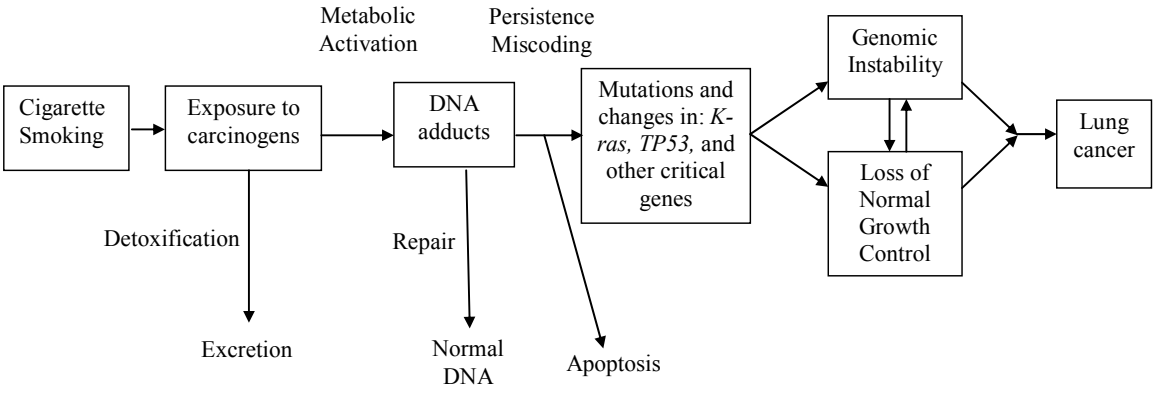

Figure 1.1 Simplified scheme linking tobacco carcinogens with lung cancer (modified from Hecht, 1999). The metabolic activation of procarcinogens in tobacco smoke results in the formation of DNA adducts, which if persistent, can lead to mutations in critical genes that can be initiators for lung tumor development. Co-carcinogens and tumour promoters also found in cigarette smoke can also contribute to lung cancer development by mechanisms that are not a result of the formation of DNA adducts. For example, these compounds can modify cell growth or apoptotic mechanisms or alter the metabolism of carcinogens that form DNA adducts.

forms in order to exert carcinogenicity. Metabolic activation (or bioactivation) results in formation of reactive intermediates which are capable of interacting with DNA and forming adducts. Cellular repair systems exist to remove DNA adducts and return DNA structure to its normal state, but if adducts escape repair and persist, mutations and/or other genomic alterations may occur. Genetic alterations in regions of crucial genes can lead to genomic instability, loss of normal cellular growth-control regulation and eventually tumor development (Hecht, 2003).

The studies conducted in this thesis have focussed on two of the key processes involved in chemical carcinogenesis, biotransformation and DNA repair, using the model pulmonary carcinogen and tobacco-specific nitrosamine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK).

1.2 CHEMICAL CARCINOGENESIS

The causal relationship between exposure to environmental substances and neoplastic development was first recognized in 1775 by the physician and surgeon, Percivall Pott, when he observed a high incidence of scrotal cancer in chimney sweeps as a result of repeated exposure to soot (Oliveira *et al.*, 2007).

A chemical carcinogen is an exogenous compound that increases the incidence of neoplasms which are defined as heritably altered, relatively autonomous growths of tissue (Pitot and Dragan, 2001). Chemical carcinogens can either be genotoxic (mutagenic) or non-genotoxic; genotoxic carcinogens act by altering the structure of DNA and/or chromosomes, while non-genotoxic carcinogens act by other mechanisms such as

modulating cell growth and cell death, or potentiating the effects of genotoxic compounds by altering cellular metabolism (Pitot and Dragan, 2001).

Chemical carcinogenesis is a multi-step, multigenic and multicausal process comprised of three stages: initiation, promotion and progression. Each of these stages are characterized by morphological and biochemical modifications that occur as a result of genetic and/or epigenetic (changes in gene expression that do not involve changes in DNA sequence) alterations (Oliveira *et al.*, 2007). The number of genes altered in a cancerous cell is not known, but it is estimated that 3 to 10 genetic events are necessary for a cell to become malignant (described below) (Barrett, 1993).

Initiation is caused by irreversible genetic alterations in genes controlling key regulatory pathways and it predisposes cells to malignant evolution and immortality. DNA damage and subsequent cell division in the absence of DNA repair is essential for the genetic alteration to become permanent (Oliveira *et al.*, 2007). Promotion is a reversible process by which the initiated cell clonally expands into preneoplastic foci; it involves cellular changes that increase the frequency of cell proliferation (Barrett, 1993). This stage does not appear to involve direct structural changes in the genome but rather depends on altered gene expression (Pitot and Dragan, 2001). During initiation and promotion, apoptosis and cell proliferation can occur but remain balanced; however, during progression, this balance shifts in favour of cell proliferation and malignancy arises (Oliveira *et al.*, 2007). During progression, cells undergo one or more additional heritable changes through genetic and/or epigenetic mechanisms to acquire a neoplastic phenotype, leading to the formation of a malignant neoplasm (Oliveira *et al.*, 2007). This

stage is characterized by irreversibility, genomic instability, accelerated growth, invasion, metastasis and changes in the biochemical, metabolic and morphological characteristics of the cells (Oliveira *et al.*, 2007).

1.2.1 Lung Cancer

Lung cancer continues to be the leading cause of cancer death in both men and women in Canada (Canadian Cancer Society, 2008) as well as the leading cause of cancer death in men and the second leading cause in women worldwide (World Health Organization, 2008). Lung cancer kills over 20,000 people annually in Canada and over 1.3 million worldwide (Canadian Cancer Society, 2008; World Health Organization, 2008). Moreover, lung cancer mortality closely parallels lung cancer incidence; 24,000 new cancer cases in Canada and 1.2 million new cases worldwide are diagnosed each year (Canadian Cancer Society, 2008; World Health Organization, 2008).

Nearly all lung cancers exhibit the morphological and molecular features of epithelial cells and are accordingly classified as carcinomas. The cells of origin of virtually all lung cancers reside in the epithelial lining of the airways. There are two main types of lung cancer, non-small cell lung cancer (NSCLC) and small-cell lung cancer (SCLC) (Schottenfeld and Searle, 2005). NSCLC accounts for approximately 80 % of all lung carcinomas and includes three main histological subtypes; squamous cell carcinoma (SCC), adenocarcinoma (AC) and large cell carcinoma (LCC), which constitute 20-25%, 30-40% and 15-20% of all lung cancers, respectively (Tuveson and Jacks, 1999; Travis *et al.*, 1995). SCC originates in the central large bronchus and often

grows more slowly than other cancer types (Schottenfeld and Searle, 2005), while AC usually originates in peripheral lung tissue including distal airways and alveoli and is the most common subtype of lung cancer (Schottenfeld and Searle, 2005). LCC is a rapidly growing form that develops throughout the lung and is characterized by the presence of large cells (Schottenfeld and Searle, 2005). SCLC is less common accounting for about 17% of lung carcinomas. It tends to arise in the larger airways, such as the primary and secondary bronchi and grows more rapidly than NSCLC. While initially more sensitive to chemotherapy than NSCLC, SCLC ultimately carries a worse prognosis and is often metastatic (Schottenfeld and Searle, 2005). Human lung tumours are often histologically heterogeneous, suggesting the presence of a common precursor cell or transitions between different histological types (Tuveson and Jacks, 1999).

Lung cancer is the most preventable of all cancers. Extensive epidemiological data has clearly established cigarette smoking as the major cause for lung cancer, with 75- 90% of all lung cancer cases being attributable to smoking (Hecht, 1999; Hecht, 2003). In addition, exposure to environmental tobacco smoke is widely accepted as a cause for lung cancer, although the risk is far lower than that of smoking (Hecht, 1999). It is important to note that tobacco use is also an important risk factor for many other cancer types such as cancers of the mouth, nasal cavity, throat, pancreas, bladder, stomach, liver and kidney and cervix (Hecht, 2003). Comprised of over 3,000 different chemicals, the complexity of tobacco smoke has made it difficult to identify the contribution of each of the more than 60 putative carcinogenic agents. These include the polycyclic aromatic hydrocarbons, *N*-nitrosamines, aromatic amines and other organic

(e.g., benzene, acrylonitrile) and inorganic (e.g., arsenic, acetaldehyde, cadmium) chemicals (Hecht, 2003).

The occurrence of lung cancer in non-smokers, who account for about 10% of cases, suggests that factors other than tobacco use are also contributing to lung cancer development. Other factors include genetics, radon gas, various workplace agents and air pollution (Schottenfeld and Searle, 2005).

There is substantial evidence for an inherited component to lung cancer. Studies of familial aggregation have demonstrated that personal lung cancer risk is associated with family incidence of lung cancer independent of smoking status (Schwartz *et al.*, 1996; Gorlova *et al.*, 2007; Etzel *et al.*, 2003), suggesting the existence of common susceptibility genes for lung cancer. The idea of a genetic contribution to lung cancer susceptibility also helps to explain the observation that only 10-15% of smokers actually develop lung cancer. Of the candidate susceptibility genes examined, many of them are involved in carcinogen activation and detoxification and DNA repair. Moreover, genetically determined polymorphisms in many of these genes are associated with lung cancer risk (Schwartz, 2005).

Inhalation of radon, which is naturally produced through the disintegration of the earth's crust, is the main source of exposure to radioactivity for most people and its decay products are readily deposited in the lung epithelium (Catelinois *et al.*, 2006). At sufficiently high concentrations, radon and its decay products cause lung cancer in smoking and nonsmoking underground uranium miners. For these individuals, their

lifetime risk of developing lung cancer is greater than 50% (Lubin *et al.*, 1995). Extrapolation of this risk to the general population which is exposed to lower levels of radon remains in question. However, chronic low level inhalation of radon in the home has been estimated to contribute to 10% of all lung cancer deaths (Lubin *et al.*, 1995).

A number of physical and chemical agents that have been identified in the workplace may also contribute to lung cancer risk. Some of the occupational agents classified as carcinogens and probable carcinogens by the International Agency for Research on Cancer include inorganic arsenic, asbestos, chromium, nickel, polycyclic aromatic compounds, radon, vinyl chloride, acrylonitrile, cadmium, formaldehyde, synthetic fibers, and silica (Schottenfeld and Searle, 2005). Currently, occupational exposures are estimated to account for 5–15% of lung cancers among men and women worldwide (Schottenfeld and Searle, 2005).

Pollutants in the urban air other than those from tobacco have been investigated as potential causal agents in the epidemic rise of lung cancer in industrialized nations (Cohen and Pope, 1995). Sources of air pollution include products of fossil fuel combustion, motor vehicle and diesel engine exhausts, power plants, and industrial and residential emissions. Potential involvement of air pollution in lung cancer has been supported by many epidemiologic studies. For instance, the ratio of urban to rural age-adjusted lung cancer mortality rates in many industrialized nations have varied between 1.1 and 2.0 and in studies of railroad workers exposed to diesel exhaust, there was a 40% increase in the smoking-adjusted relative risk of lung cancer (Garshick *et al.*, 1988).

1.2.2 Molecular Alterations in Human Lung Cancer

During lung cancer development persistent genetic lesions which occur as a result of both genetic and epigenetic alterations, accumulate at specific chromosomal loci, increasing genomic instability and driving the progressive transformation of normal cells into highly malignant ones (Hanahan and Weinberg, 2000). Genetic alterations include point mutations, amplifications, deletions and translocations, while common epigenetic alterations include changes in promoter methylation or histone acetylation status. These aberrations are required for the inactivation of tumour suppressor genes and the activation of proto-oncogenes, events believed to be essential in chemical carcinogenesis. Moreover, inactivation of the *TP53* tumour suppressor gene and activation of the K-*ras* proto-oncogene are common events in many types of human cancers including lung cancer.

The tumour suppressor protein, P53, is a nuclear phosphoprotein that is involved in many critical cellular processes including gene transcription, DNA synthesis and repair, genomic plasticity, cell cycle control and apoptosis (Greenblatt *et al.*, 1994). Mutation of *TP53* (mouse ortholog, *Trp53*) is the most common genetic lesion in human cancers (Harris, 1995) and is mutated in over two-thirds of lung cancers (Bennett *et al.*, 1993). *TP53* mutations, predominantly representing G to T transversions (a purine to pyrimidine exchange), occur in 70% of SCLC and 47% of NSCLC, including 65% of SCC, 60% of LCC and 33% of AC (Greenblatt *et al.*, 1994; Johnson and Kelley, 1993). *TP53* mutations play a key role in tumour development since loss of P53 causes dysregulation of cell-cycle control and stimulates cell growth. *TP53* mutations occur

preferentially on the non-transcribed strand of the *TP53* gene and major mutational "hotspots" have been identified in lung tumours, occurring at codons, 157, 158, 245, 248, 249, 273 (Greenblatt *et al.*, 1994; Pfeifer *et al.*, 2002). Although *TP53* is believed to be an important early target for mutations in human lung cancers, there does not appear to be an association between the presence of this mutation and patient survival (Li *et al.*, 1994; Johnson and Kelley, 1993).

K-*ras* is a proto-oncogene and a member of the *ras* gene family (H-*ras*, K-*ras*, N-*ras*). These genes encode guanine triphosphate-binding proteins that are involved in cell growth control. K-ras transmits signals from membrane-bound growth factor receptors to activate intracellular signaling pathways, leading to alterations in transcription factors and cell cycle proteins involved in cell proliferation and differentiation (Boguski and McCormick, 1993). K-*ras* mutations, most frequently a G to T tranversion in codon 12, are seen in 30% of lung AC (Westra *et al.*, 1993), less commonly in other lung cancer subtypes and almost never in SCLC (Meuwissen and Berns, 2005). Since K-ras normally positively regulates cell proliferation, K-*ras* mutations are important in tumour development as mutations can cause constitutive activation of K-ras leading to uncontrolled cell proliferation and differentiation. Because K-*ras* mutations are found early in human alveolar atypical hyperplasia, a presumed precursor lesion to AC (Cooper *et al.*, 1997), it is believed that this may be an important step in the initiation of this subtype of lung cancer. Moreover, the presence of K-*ras* mutations has been demonstrated to be an adverse prognostic factor for survival in patients with NSCLC (Johnson and Kelley, 1993).

1.2.3 Modelling Lung Cancer in the Mouse

Currently, several animal species are widely used for experimental lung cancer research including dogs, primates, hamsters, rats and mice (Coggins, 2001). Among these, mice have become the preferred model with which to study lung cancer development and progression. This is largely due to fact that primary lung tumours in mice have morphologic, histogenic, and molecular features similar to those of human lung AC (Malkinson, 2001; Malkinson, 1992; Malkinson, 1998). In addition, the high degree of genetic homology between human and mouse, the ease of genetic manipulation, and the large number of genetically altered mice available for experimentation also contribute to their usefulness (Malkinson, 2001). By characterizing the processes involved in lung cancer development, mouse models can serve as valuable tools for understanding basic lung tumour biology, for the development and validation of new therapeutic interventions and for the identification of markers for early diagnosis (Meuwissen and Berns, 2005).

In contrast to most other species which develop various different histological subtypes of lung cancer, mice develop mainly AC (Malkinson, 1998). The stages involved in AC development are thought to be identical in mouse and human. The sequence of tumour progression is hyperplasia → adenoma → carcinoma *in situ* → metastatic carcinoma (Malkinson, 1998). Although each stage has been identified in both species, disparity exists in the degree to which each of these stages has been studied and characterized. The mouse model has been used mainly to study the early changes and processes of AC development including hyperplasias and adenomas. Advanced tumours

and metastases rarely occur in mice because malignant growth in lung usually causes respiratory distress and death (Malkinson, 1998). In contrast, most information about human AC comes from late-stage metastatic carcinomas as this is usually the stage when diagnosis occurs (Malkinson, 1998; Tuveson and Jacks, 1999). In both humans and mice, it has been suggested that bronchiolar non-ciliated Clara cells and alveolar type II cells are the cells of tumour origin (Malkinson, 2001; Thaete and Malkinson, 1991).

The susceptibility of mice to developing spontaneous- or chemically-induced lung cancer varies among inbred strains. Mouse strains have been categorized into sensitive (A/J, SWR), intermediate (BALB/c, O20), relatively resistant (CBA, C3H) and resistant (C57BL/6, DBA), (Tuveson and Jacks, 1999; Meuwissen and Berns, 2005) based on latency time for lung tumour appearance and on tumour number (Malkinson, 2001). The relative propensity of these strains to develop lung tumors is strongly linked to the pulmonary adenoma susceptibility 1 (*Pas1*) locus on chromosome 6 (Manenti and Dragani, 2005). Several genes have been proposed as candidates for the *Pas1* locus including K-*ras*. Predisposition to lung tumour development strongly correlates with a polymorphism in the second intron of K-*ras* (You *et al.*, 1992); this polymorphism is a 37 bp sequence that is tandemly duplicated in resistant strains and is present as a single copy in sensitive strains, and is believed to influence K-ras expression (Malkinson and You, 1994; Chen *et al.*, 1994). In addition to *Pas1*, at least 12 other *Pas* loci as well as *Par* (pulmonary adenoma resistance) and *Sluc* (susceptibility to adenomas resistance) loci have been mapped within the mouse genome and are thought to carry susceptibility genes that influence lung tumour development (Meuwissen and Berns, 2005; Tuveson and

Jacks, 1999). Moreover, most of the human chromosomal regions homologous to the mouse lung tumour susceptibility sites are frequently altered in NSCLC, implying that they are also important in regulating human lung cancer development (Malkinson, 2001).

It is estimated that 30-50% of all human lung AC have mutations in codons 12,13 or 61 of K-*ras* while other human lung cancer types rarely have these mutations (Tuveson and Jacks, 1999; Malkinson, 2001). Similarly, >90% of spontaneous- and chemically-induced mouse lung tumours display a variety of base substitutions in these same K-*ras* codons (Malkinson, 2001; Malkinson, 1998). The type (base transversion or transition) and location of mutation within a particular codon depends on the type of carcinogen DNA-adduct formed. In addition, the age of the mouse at the time of carcinogen exposure, the carcinogen dose and the mouse strain can also influence the K-*ras* mutational spectrum (Malkinson, 2001). Similar to human (Li *et al.*, 1994; Rodenhuis and Slebos, 1990), point mutations in K-*ras* is a very early event in mouse, suggesting that this mutation may be important in lung tumourigenesis in both species (Anderson *et al.*, 1992; Malkinson, 2001; Malkinson, 1998).

Later genetic events in mouse lung tumourigenesis involve tumour suppressor genes. Many of these genes are initially inactivated by methylation in hyperplasia and adenomas but deleted within carcinomas. Examples of such genes include those which encode for cell-cycle regulatory proteins, p16 and Rb (Malkinson, 2001; Malkinson, 1998). Many of these genes are commonly hypermethylated and deleted in human AC, although the frequency of tumour suppressor gene inactivation is lower in mouse lung tumours than in human lung tumours because these are generally late events (Malkinson,

2001). Loss of P53 is much more common in human AC than in mouse AC where it is found infrequently and only in more advanced lesions (Tuveson and Jacks, 1999; Malkinson, 1998; Tam *et al.*, 1999).

1.3 4-(METHYLNITROSAMINO)-1-(3-PYRIDYL)-1-BUTANONE (NNK)

1.3.1 Formation and Sources

Tobacco contains a number of different alkaloids including nornicotine, anabasine, anatabine and nicotine and among these tobacco alkaloids, nicotine is found in the highest amounts (Hecht and Hoffmann, 1988). These tobacco alkaloids undergo nitrosation to form chemical compounds collectively known as tobacco-specific nitrosamines (TSNAs). Seven TSNAs, *N*'-nitrosonornicotine (NNN), the nicotine-derived nitrosaminoketone (NNK), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), *N*'-nitrosoanatabine (NAT), *N*'-nitrosoanabasine (NAB), 4-(methylnitrosamino)-4-(3-pyridyl)-1-butanol (*iso*-NNAL), and 4-(methylnitrosamino)-4-(3-pyridyl)butyric acid (*iso*-NNAC) have been identified (Figure 1.2). While most are present in both unburned tobacco and tobacco smoke (Hecht and Hoffmann, 1988; Brunnemann and Hoffmann, 1991; Hecht, 1998), NNN, NNK and NAT are found in the highest quantities (Hecht, 1998). Nicotine is a significant precursor for TSNAs, forming NNK, NNN and the nicotine-derived nitrosaminoaldehyde, NNA which can be reduced to form iso-NNAL (Hecht *et al.*, 1978b; Hecht *et al.*, 1978a). Of all TSNAs, the nicotine-derived TSNAs, NNN, NNK and NNAL are also the most carcinogenic (Hecht, 1998).

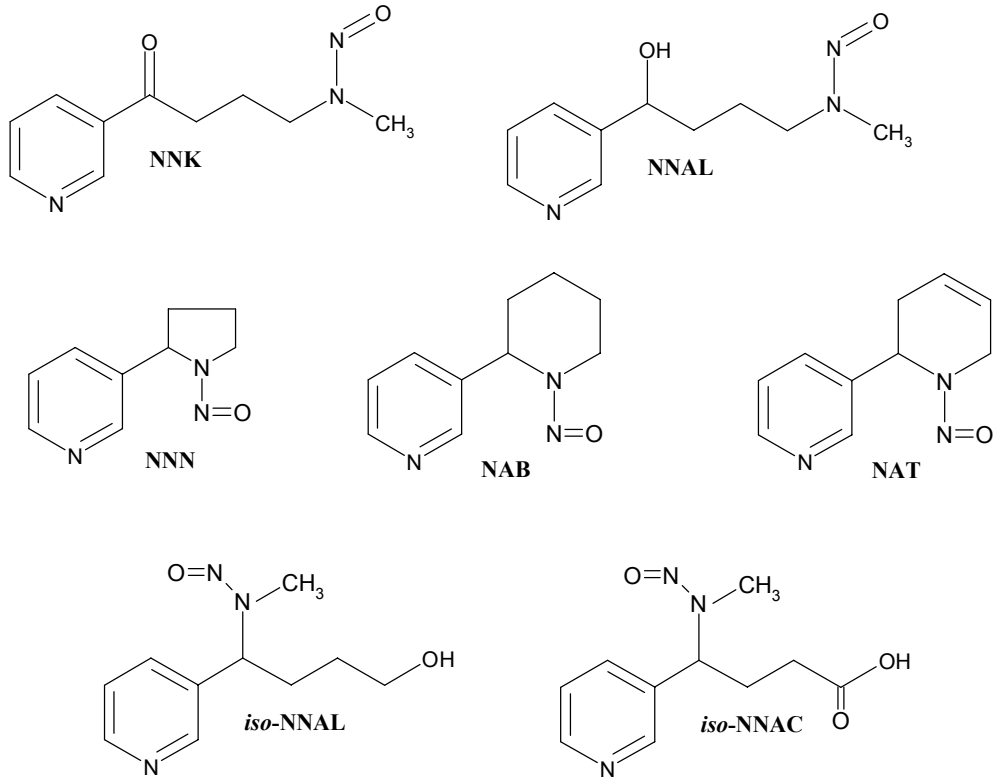

Figure 1.2 Structures of tobacco-specific nitrosamines formed by the nitrosation of tobacco alkaloids. Abbreviations stated in text.

The formation of NNN, NNA and NNK from the reaction of a nitrosating agent with nicotine has been demonstrated *in vitro* (Hecht *et al.*, 1978b). This same process can occur as a result of metabolism within the tobacco plant, during the curing, processing and burning (smoking) of tobacco. It is thought that additional amounts of NNK and other TSNAs are also formed *in vivo* from the reaction of nicotine with different nitrosating species such as nitrite present in saliva, nitrogen oxides present in inhaled mainstream tobacco smoke and possibly nitric oxide synthase (Hecht and Hoffmann, 1988; Nair *et al.*, 1996). Alternatively, human liver microsomes and cytochrome P450 2A6 have been shown to catalyze the 2′-hydroxylation of nicotine, leading to formation of NNK (Hecht *et al.*, 2000).

1.3.2 Carcinogenicity of NNK in Experimental Animals

NNK is the most prevalent pulmonary carcinogen in tobacco smoke and the most potent cancer-causing TSNA in all animal species tested (Hecht, 1998). NNK is a potent lung carcinogen in rodents, and selectively induces lung AC regardless of its route of administration (Hecht, 1998). Thus, when NNK is applied topically to the skin, taken orally or injected by intraperitoneal, subcutaneous or intravesical routes, lung tumours develop (Hoffmann *et al.*, 1996).

In rat lung, tumours are thought to arise from alveolar type II cells and are predominantly adenomas and ACs, although a low incidence of SCCs have been observed (Hecht, 1998). Lung tumours are prevalent at a wide range of NNK doses including lower doses where tumours are absent in other areas (Rivenson *et al.*, 1988;

Belinsky *et al.*, 1990). At higher doses of NNK, liver tumours are also observed following subcutaneous injection (Belinsky *et al.*, 1990; Hoffmann *et al.*, 1984) and when administered in drinking water, NNK induces low levels of exocrine pancreatic tumours (Rivenson *et al.*, 1988). Moreover, NNK and its metabolite NNAL are the only tobacco constituents known to induce exocrine pancreatic tumours (Rivenson *et al.*, 1988).

NNK treatment results predominantly in the development of lung tumours in both sensitive and resistant mouse strains, although tumour incidence and multiplicity are usually lower in resistant strains (Hecht, 1998). In addition, although rare, liver and forestomach tumours can also occur. The A/J strain is the mouse model that has been used most extensively for NNK carcinogenicity studies. In the A/J mouse, a single intraperitoneal dose of 10 μmol NNK results in significant tumour induction after 16 weeks, producing 7-12 lung tumours per mouse (Hecht *et al.*, 1989). After 14 weeks, hyperplasias, apparently arising from type II cells, can be observed along the alveolar septa (Hecht, 1998). Inhibitors of tumour formation have also been studied in this mouse strain. Isothiocyanates and indole-3-carbinol inhibit lung tumour induction by NNK, most likely as a result of alterations in NNK bioactivation (Hecht, 1998). In contrast to the rat, in which NNK and NNAL are equipotent lung tumourigens (Rivenson *et al.*, 1988), in A/J mice, NNAL has 30-70% the potency as NNK for lung tumour induction (Castonguay *et al.*, 1983; Hecht *et al.*, 1990; Hoffmann *et al.*, 1993).

In the hamster, the lung, trachea, and nasal cavity are the main target tissues of NNK (Hecht, 1998). Respiratory tumours are induced by many different NNK dosing protocols when administered either subcutaneously or when applied to the cheek pouch

(Hecht, 1998). Unlike in the mouse, NNK is a potent transplacental carcinogen in the hamster, inducing tumours of the respiratory tract and adrenal glands in the offspring following injection of NNK during gestation (Hecht, 1998).

1.3.3 NNK and Linkage to Human Cancers

Tobacco products contain a wide array of chemical carcinogens that cause cancers of various types. There are more than 60 known carcinogens in tobacco smoke and at least 16 in unburned tobacco (Hecht, 2003). However, among these carcinogens, NNK seems to play a particularly important role in cancer induction due to its carcinogenic activity and the fact that it is one of the most prevalent carcinogens in tobacco smoke (Hecht, 1998). NNK has been classified as being carcinogenic to humans (Group I carcinogen) according to the International Agency for Research on Cancer (International Agency for Research on Cancer, 2008).

The organospecificity of NNK for the lung provides strong support for its potential role in lung cancer induction by tobacco smoke. NNK targets the lung in all animals tested, independent of the route of administration, (Hoffmann *et al.*, 1996). Doses of NNK that are effective in producing lung tumours in laboratory animals are comparable to the estimated levels of NNK exposure in long-term smokers (Hoffmann *et al.*, 1996). For instance, the cumulative dose of 1.8 mg/kg of NNK that induces a significant number of lung tumours in rats (Belinsky *et al.*, 1990) is similar to the cumulative lifetime dose of 1.6 mg/kg that is estimated for the average individual who smoked 2 packs a day for 40 years (Hecht and Hoffmann, 1989; Hecht, 1998). In

humans, NNK metabolites are found in the urine of people that use or are exposed to tobacco products (Hecht, 2002) and DNA adducts derived from NNK are present at higher levels in lung tissue from lung cancer patients than from controls (Schlobe *et al.*, 2002), suggesting that individuals are capable of activating NNK and forming DNA adducts selectively in the lung, respectively.

NNK is believed to be responsible for the changing histology of lung cancer. Since the 1950s, the leading lung cancer subtype has switched from SCC to AC and this is consistent with a role for NNK which produces primarily AC in rodents (Hecht, 2003). The alterations in histological patterns coincide with changes in the composition and design of cigarettes and in smoking behavior (Thun *et al.*, 1997; Hoffmann *et al.*, 1996). The amounts of NNK in mainstream smoke have increased as the nitrate levels in tobacco increased from 1959 to 1997 because of the use of higher amounts of air-cured tobacco in the tobacco blends (Hoffmann *et al.*, 2001). In addition, because of the introduction of lower nicotine yield filtered cigarettes since the 1950s, smokers were found to smoke more intensely and inhale more deeply than smokers of unfiltered plain cigarettes, thus transporting tobacco carcinogens such as NNK deeper into the peripheral lung (Thun *et al.*, 1997; Hoffmann *et al.*, 1996).

There are several lines of evidence that indicate that NNK has a major role in the causation of oral cancer in snuff-dippers. NNK and NNN are by far the most prevalent carcinogens in unburned tobacco products such as oral snuff, chewing tobacco and other smokeless tobacco products, (Hecht, 2003) and epidemiological studies have demonstrated that snuff-dipping causes oral cancer. In addition, NNK and NNN are the

only known carcinogens in snuff that produce oral tumours in laboratory animals (Preston-Martin, 1991), and the levels of total exposure to NNK experimentally are similar to those estimated for a long-term snuff-dipper (Hecht and Hoffmann, 1988). Also, NNK is present in the saliva of snuff dippers and human and animal buccal tissue can activate NNK into DNA-binding intermediates (Preston-Martin, 1991).

In addition to cancers of the lung and oral cavity, both cigarette smoking and NNK are causally associated with other cancer types (Hecht, 2003). NNK treatment produces tumours in the esophagus, nasal cavity and pancreas in some laboratory animals (Hecht and Hoffmann, 1988). In fact, NNK and its major metabolite NNAL are the only pancreatic carcinogens that are known to be present in tobacco products, and biochemical studies from human tissues support their involvement in smoking-related pancreatic cancer (Hecht, 2003).

1.4 BIOTRANSFORMATION OF NNK

1.4.1 Metabolic Pathways

NNK metabolism pathways are illustrated in Figure 1.3. The main pathways include carbonyl reduction, α-hydroxylation (hydroxylation of the carbons adjacent to the *N*-nitroso group), and pyridine *N*-oxidation (Hecht, 1998).

Carbonyl reduction of NNK results in the formation of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL). NNAL is not a detoxified metabolite of NNK since NNAL can undergo similar bioactivation pathways as NNK. In contrast to NNK, NNAL can be glucuronidated to form both β-*O*-[4-(methylnitrosamino)-1-(3-pyridyl)-1-but-1-

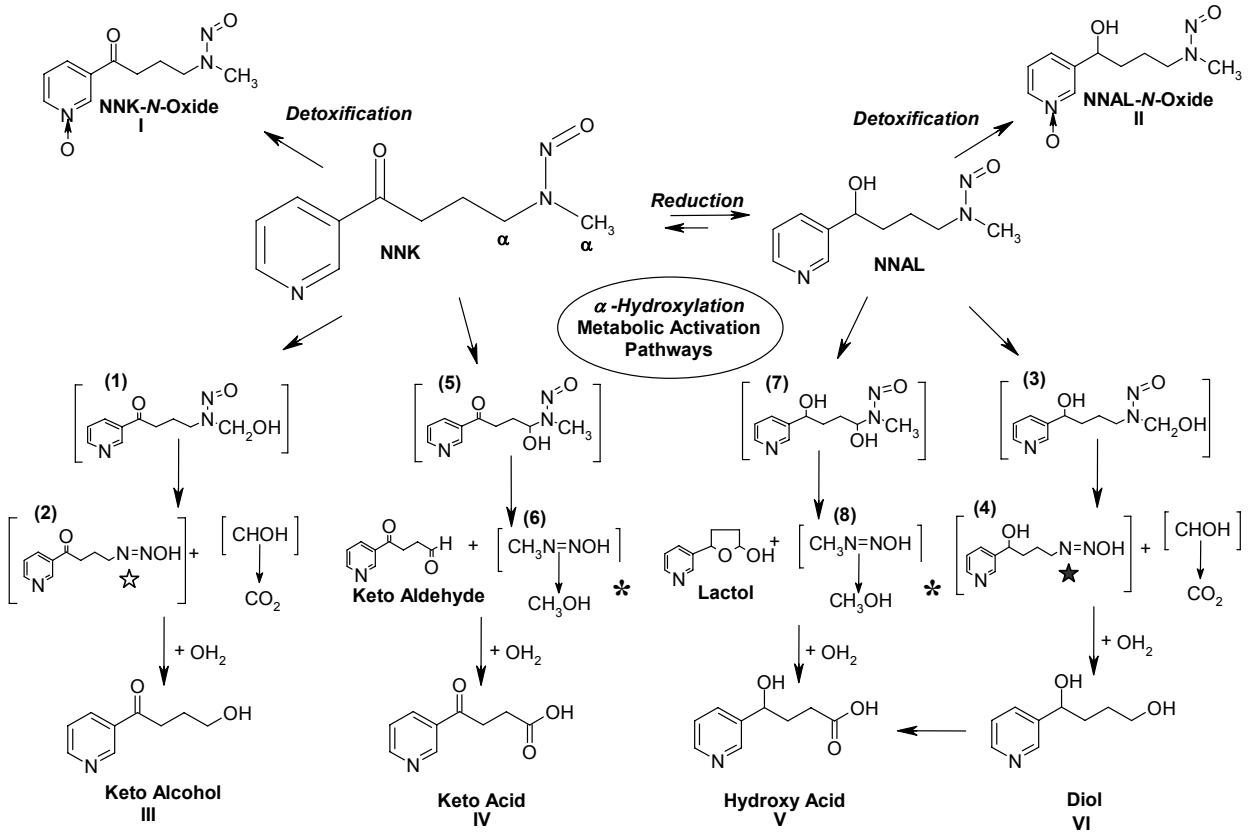

Figure 1.3 Pathways of NNK metabolism. Metabolites I and II are detoxification products produced by pyridine *N*-oxidation; metabolites III to VI are endpoint products of α-hydroxylation and are indicative of the formation of unstable reactive metabolites which methylate * , pyridyloxobutylate ☆ , or pyridylhydroxybutylate ★ DNA.

yl]-D-glucosiduronic acid (NNAL-*O*-Glu) if glucuronidation occurs at the carbinol group (Morse *et al.*, 1990; Carmella *et al.*, 1993; Ren *et al.*, 2000) and β-*N*-[4-(methylnitrosamino)-1-(3-pyridyl)-1-but-1-yl]-D-glucosiduronic acid (NNAL-*N*-Glu) if glucuronidation occurs on the pyridine nitrogen (Carmella *et al.*, 2002; Wiener *et al.*, 2004). Both NNAL-*O*-Glu and NNAL-*N*-Glu are thought to be important detoxification products of NNK since they are readily excreted. NNAL can be oxidized to NNK and this reaction may be important since it is thought that the carcinogenicity of NNK may be partly attributable to the reconversion of NNAL to NNK (Hecht, 1998).

To exert carcinogenicity, both NNK and NNAL are bioactivated by α-carbon hydroxylation to form DNA-reactive species via two different pathways. α-Methyl carbon hydroxylation of NNK generates 4-(hydroxymethylnitrosamino)-1-(3-pyridyl)-1-butanone **(1)**, which decomposes to 4-oxo-4-(3-pyridyl)-1-butanediazohydroxide **(2)** and formaldehyde. Compound **2** pyridyloxobutylates DNA and the reaction of **2** with water produces 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB or keto alcohol) (**III**). In addition, **2** undergoes *O*-glucuronidation in rats and the resulting glucuronide is potentially an important detoxification product of NNK (Murphy *et al.*, 1995). α-Methyl carbon hydroxylation of NNAL forms a similar intermediate **(3)**, ultimately generating the DNA pyridylhydroxybutylating species, 4-hydroxy-4-(3-pyridyl)-1-butanediazohydroxide **(4)** and 4-hydroxy-1-(3-pyridyl)-1-butanol (diol) (**VI**).

α-Methylene carbon hydroxylation of NNK leads to formation of 4-hydroxy-4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone **(5)**, which spontaneously decomposes to

methane diazohydroxide **(6)** and keto aldehyde. Methane diazohydroxide methylates DNA, while keto aldehyde is further oxidized to form 4-oxo-4-(3-pyridyl) butyric acid (keto acid) (**IV**). α-Methylene carbon hydroxylation of NNAL also forms the methylating species, methane diazohydroxide **(8)** and a hydroxy aldehyde which cyclizes to lactol and is further oxidized to 4-hydroxy-4-(3-pyridyl) butyric acid (hydroxy acid) (**V**).

In order to assess experimentally the degree of NNK and NNAL bioactivation by α-carbon hydroxylation, formation of the four endpoint metabolites, keto acid, keto alcohol, hydroxy acid and diol are often used since their formation is indicative of the formation of the DNA-reactive metabolites. NNK-induced DNA adducts are not measured directly since they are formed at below quantifiable levels.

The detoxification of NNK and NNAL occurs mainly through pyridine *N*-oxidation, which results in the formation of excretable *N*-oxides (Hecht, 1998). Pyridine *N*-oxidation of NNK and NNAL produces 4-(methylnitrosamino)-1-(3-pyridyl *N*-oxide)-1-butanone (NNK-*N*-oxide) and 4-(methylnitrosamino)-1-(3-pyridyl *N*-oxide)-1-butanol (NNAL-*N*-oxide), respectively.

1.4.2 Cytochrome P450-Mediated NNK Biotransformation

Cytochrome P450 enzymes (CYP), particularly those in the CYP*1*, CYP*2*, and CYP*3* gene families catalyze the biotransformation of a wide variety of xenobiotics including carcinogens. These microsomal enzymes are found in the highest concentrations in the liver but are also present in most other tissues including the lung.

The majority of studies have focussed on the reactions involved in the bioactivation of NNK and these studies suggest that CYPs are the major enzymes involved (Hecht, 1998).

Several human CYPs have been implicated in NNK metabolism and are summarized in Table 1.1, although the major enzyme(s) involved in human lung have yet to be elucidated. Studies aimed at determining which CYP isozymes are responsible for NNK metabolism have focussed on CYP enzymes that are efficient catalysts of NNK bioactivation (i.e. have high V_{max} and low K_M values) since the *in vivo* concentration of NNK in humans is likely very low (pM to low nM)(Hecht, 1998; Jalas *et al.*, 2005). Based on *in vitro* V_{max} and K_M data, the relative efficiencies by which the different CYPs catalyze NNK metabolism are (from highest to lowest): 2A13 > 2B6 > 2A6 > 1A2 ≈ 1A1 > 2D6 ≈ 2E1 ≈ 3A4 (Jalas *et al.*, 2005). However, *in vivo*, the involvement of each of these enzymes in NNK metabolism is dependent on various factors including relative expression levels, the amount of NADPH-cytochrome P450 oxidoreductase expressed, intra-tissue localization, enzyme inducibility, and the concentration of NNK in that tissue.

In human liver, based on expression and kinetic studies, CYPs 1A2, 2A6, 2B6 and 3A4 have been implicated as the main CYPs responsible for NNK α-hydroxylation (Patten *et al.*, 1996; Staretz *et al.*, 1997b), but the relative contribution of these enzymes is likely to vary among individuals.

In human lung, evidence has accumulated supporting roles for CYP1A1, 1A2, 2A6, 2A13, 2B6, 2D6, 2E1, and 3A4 in NNK metabolism (Smith *et al.*, 1992a; Smith *et al.*, 1995; Hecht, 1998; Smith *et al.*, 1999; Smith *et al.*, 2003; Jalas *et al.*, 2005; Ding and

Table 1.1 Cytochrome P450 enzymes involved in human NNK biotransformation

P450 isoform	Metabolism	Tissue Expression	References
1A1	α-Methyl hydroxylation α-Methylene hydroxylation	Induced in many tissues including the lung and liver	(Shimada *et al.*, 1994; Wei *et al.*, 2002; Raunio *et al.*, 1999; Ding and Kaminsky, 2003)
1A2	α-Methyl hydroxylation α-Methylene hydroxylation	Constitutively expressed in liver; may be induced in lung	(Shimada *et al.*, 1994; Wei *et al.*, 2002; Smith *et al.*, 1996; Smith *et al.*, 1992a; Ding and Kaminsky, 2003)
2A6	α-Methyl hydroxylation α-Methylene hydroxylation *N*-oxidation	Expressed at relatively low levels in liver and lung	(Patten *et al.*, 1996; Shimada *et al.*, 1996; Crawford *et al.*, 1998; Smith *et al.*, 2003; Ding and Kaminsky, 2003)
2A13	α-Methyl hydroxylation α-Methylene hydroxylation *N*-oxidation	Lung and nasal cavity	(Su *et al.*, 2000; Zhu *et al.*, 2006; Zhang *et al.*, 2002; Jalas *et al.*, 2003; Smith *et al.*, 2003)
2B6	α-Methyl hydroxylation α-Methylene hydroxylation *N*-oxidation	Expressed at low levels in liver and lung	(Dicke *et al.*, 2005; Shimada *et al.*, 1996; Smith *et al.*, 2003; Raunio *et al.*, 1999)
2D6	α-Methyl hydroxylation α-Methylene hydroxylation	Liver; other extrahepatic tissues including the lung	(Patten *et al.*, 1996; Guidice *et al.*, 1997; Ding and Kaminsky, 2003)
2E1	α-Methyl hydroxylation α-Methylene hydroxylation *N*-oxidation	Liver; other extrahepatic tissues including the lung	(Hukkanen *et al.*, 2002; Patten *et al.*, 1996; Smith *et al.*, 2003; Ding and Kaminsky, 2003)
2F1	α-Methyl hydroxylation α-Methylene hydroxylation	Selectively expressed in lung	(Nhamburo *et al.*, 1990; Smith *et al.*, 1992a)
3A4	α-Methyl hydroxylation α-Methylene hydroxylation *N*-oxidation	Liver; other extrahepatic tissues including the lung	(Patten *et al.*, 1996; Jalas *et al.*, 2005; Raunio *et al.*, 1999; Ding and Kaminsky, 2003)

Kaminsky, 2003). However, recently it has been suggested that the CYP2A enzymes may play a particularly large role in pulmonary NNK metabolism because of their relatively high catalytic efficiency for NNK bioactivation and their substantial expression in human lung. CYP2A13 mRNA and protein are expressed predominantly in the human respiratory tract including the nasal mucosa, trachea and peripheral lung (Su *et al.*, 2000) and the protein is expressed at high levels within epithelial cells of the human bronchus and trachea (Zhu *et al.*, 2006). Heterologously-expressed CYP2A13 exhibits higher catalytic activity for NNK activation than do other CYP isoforms examined (Su *et al.*, 2000; Smith *et al.*, 1992a; Patten *et al.*, 1996), having the lowest K_M value and the highest V_{max}/K_M values for NNK metabolism (Su *et al.*, 2000). The α-hydroxylation of NNAL can also be catalyzed by recombinant CYP2A13 but with less efficiency than the α-hydroxylation of NNK (Jalas *et al.*, 2003). The other CYP2A isozyme, CYP2A6, is the main CYP2A isoform in liver (Su *et al.*, 2000) but it is also present in human nasal mucosa, trachea and lung (Su *et al.*, 2000). Compared to other CYP isozymes, CYP2A6 is also one of the most efficient catalysts of NNK α-hydroxylation (Jalas *et al.*, 2005).

Because of their selective expression in human lung, CYPs 2S1 (Rylander *et al.*, 2001), and 4B1, (Nhamburo *et al.*, 1989) may be of particular interest in terms of their involvement in NNK metabolism. While the role of CYP4B1 remains to be elucidated, recently it has been suggested that NNK is not a substrate for CYP2S1 (Wang *et al.*, 2005b).

There is relatively little information regarding the role of CYP enzymes in NNK detoxification by pyridine *N*-oxidation. CYP3A4 may be involved as CYP3A4 activity

was correlated with NNK and NNAL *N*-oxide formation in human liver microsomes (Hecht *et al.*, 1983). A study of NNK metabolism in human lung microsomes implicated CYP2A6, 2B6 and 2E1 as important contributors to *N*-oxide formation (Smith *et al.*, 2003). Consistent with a role of CYP2A enzymes, recently it was demonstrated that CYP2A13 could catalyze the *N*-oxidation of NNAL but not of NNK (Jalas *et al.*, 2003).

NNK metabolism has been studied in the rabbit, hamster and the patas monkey but most extensively in the rat and mouse (Jalas *et al.*, 2005; Hecht, 1998). In rat, CYPs 1A1, 1A2, 2A3, 2B1 and 2C6 may be involved in NNK metabolism. CYP2A3 is expressed in both the nasal mucosa and the lung but not in the liver (Su *et al.*, 1996) and shares an 85% and 87 % sequence homology with CYP2A6 and CYP2A13, respectively (Jalas *et al.*, 2005). In addition, CYP2A3 catalyzes NNK α-hydroxylation with a similar efficiency to CYP2A13 (Jalas *et al.*, 2003), suggesting that this isozyme may be important in NNK-induced carcinogenicity in rat lung. While all of the other CYP isozymes catalyze NNK α-hydroxylation, CYP2B1 and CYP2C6 can also catalyze the *N*-oxidation of NNK (Guo *et al.*, 1991; Lacroix *et al.*, 1993) which is of importance as NNK-*N*-oxide is generally the major metabolite formed in rat and mouse lung (Hecht, 1998).

In mouse, CYP2A4 and CYP2A5 have demonstrated involvement in NNK bioactivation. These enzymes are expressed in many tissues including lung and liver and have a greater than 90 % sequence homology with human CYP2A6 and CYP2A13 (Honkakoski and Negishi, 1997). Similar to the other CYP2A enzymes, mouse CYP2A5 also has high catalytic efficiency for the α-hydroxylation of NNK (Jalas *et al.*, 2003).

1.4.3 Other NNK Biotransformation Enzymes

CYP protein level and activity in human lung are considerably lower than in human liver (Jalas *et al.*, 2005; Ding and Kaminsky, 2003; Smith *et al.*, 1991). In fact, it has been estimated that CYP levels in whole lung microsomes are between 1 and 10 pmol/mg protein (Shimada *et al.*, 1992) and about 344 ± 167pmol/mg protein in hepatic microsomes (Shimada *et al.*, 1994). While CYPs are expressed in particular regions of the lung and within certain cell types, which results in higher localized concentrations (Anttila *et al.*, 1997; Hukkanen *et al.*, 2002), it is possible that non-CYP enzymes may be important in NNK metabolism in extrahepatic tissue such as the lung where CYP levels are much lower.

CYP-independent pathways involving prostaglandin H synthase (PHS) and lipoxygenase (LOX) have been suggested to play a role in NNK metabolism in human lung (Smith *et al.*, 1995). Both PHS and LOX are found at high levels relative to CYPs in the lung (Hamberg *et al.*, 1980; Ochiai *et al.*, 1999) and inhibitors of PHS and LOX have been reported to decrease NNK metabolism (Smith *et al.*, 1995) and NNK-induced lung tumour incidence (Rioux and Castonguay, 1998). Although PHS-mediated NNK biotransformation remains to be more fully investigated, it has been demonstrated that LOX is unable to bioactivate NNK in human lung (Bedard *et al.*, 2002).

PHS normally mediates the oxidation of arachidonic acid in the production of prostaglandins as a result of its cyclooxygenase and peroxidase activities. Both of these activities can contribute to carcinogen bioactivation. During arachidonic acid metabolism as a result of PHS peroxidase activity, xenobiotics can undergo cooxidation, a process

whereby the reduction of arachidonic acid occurs concomitantly with the oxidation of the xenobiotic, forming reactive intermediates (Krauss and Eling, 1984; Eling and Curtis, 1992). As well leakage of peroxyl radicals occurs during PHS cyclooxygenase reactions and these radicals interact with and oxidize such carcinogens as benzo[a]pyrene (Pruess-Schwartz *et al.*, 1989) and aflatoxin B_1 (Marnett, 1990). However, it is likely that only the peroxidase activity of PHS is important in NNK bioactivation since peroxyl radicals fail to bioactivate NNK (Bedard *et al.*, 2002).

Another important reaction in NNK metabolism is the carbonyl reduction of NNK to form its major metabolite NNAL. It is thought that CYPs are not the major catalysts of NNAL formation but rather that this reaction is mediated by carbonyl reductases (Hecht, 1998). More specifically, microsomal 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) (Maser, 1998; Maser *et al.*, 1996; Finckh *et al.*, 2001) and cytosolic carbonyl reductase (EC 1.1.1.184) (Maser *et al.*, 2000; Atalla *et al.*, 2000; Finckh *et al.*, 2001), which both belong to the protein superfamily of short-chain dehydrogenases/reductases, are believed to be the major contributors to this conversion. In microsomes, 11β-HSD1 catalyzes the carbonyl reduction of NNK to form NNAL in both human and mouse lung and liver (Maser *et al.*, 2000; Soldan *et al.*, 1999; Atalla and Maser, 1999), while in human and mouse lung and liver cytosols, carbonyl reductase is responsible for NNK-carbonyl reductase activity (Maser *et al.*, 2000; Atalla *et al.*, 2000; Finckh *et al.*, 2001).

It is likely that enzymes other than 11β-HSD1 and carbonyl reductase participate in NNK reduction in human tissues. Studies have not been able to show a clear correlation between interindividual differences in either 11β-HSD1 or carbonyl reductase

expression and NNK carbonyl reductase activity in human lung microsomes and cytosols, respectively (Finckh *et al.*, 2001; Soldan *et al.*, 1999), and experiments with an 11β-HSD inhibitor indicated a minor or no role for 11β-HSD (Breyer-Pfaff *et al.*, 2004). In fact, cytosolic enzymes belonging to the ARK1C superfamily of aldo-ketoreductases catalyze NNK carbonyl reduction, albeit with lower efficiency than carbonyl reductase (Atalla *et al.*, 2000). In addition, although it has been suggested that CYPs are not the major catalysts of NNAL formation (Hecht, 1998), the fact that this reaction is highly dependent upon the presence of an NADPH-generating system (Maser *et al.*, 2000; Smith *et al.*, 2003) and is sensitive to inhibition by CYP inhibitors (Smith *et al.*, 2003), suggests a possible role.

The glucuronation of NNAL is catalyzed by UDP-glucuronosyltransferases (UGTs) in both humans and rodents. The majority of NNAL-*O*-Glu formation occurs in the liver and is thought to be mediated mainly by UGTs 1A9 and 2B7 in humans (Ren *et al.*, 2000). Although to date the glucuronidation of NNAL has not been observed in human lung, pulmonary NNAL-*O*-Glu formation may occur since lung does have glucuronidation activity and detectable levels of UGT2B7 (Ren *et al.*, 2000). NNAL-*N*-Glu formation occurs in human liver microsomes and UGTs 1A4 and 2B10 catalyze this reaction (Wiener *et al.*, 2004; Chen *et al.*, 2008). In rats, the major UGT enzyme involved in NNAL-*O*-Glu formation is UGT2B1 with no involvement of the UGT1 family (Ren *et al.*, 1999).

1.4.4 NNK Biotransformation in Experimental Animal Lung

In vitro NNK metabolism in rat, hamster and mouse, rabbit, pig and monkey has mainly been studied using microsomes. However, cultured tissues and cells, cytosols and freshly isolated lung cell types have also been used from liver, lung, nasal mucosa, oral tissue, trachea, esophagus, stomach, intestine, and pancreas (Hecht, 1998). *In vivo* NNK metabolism studies have also been carried out in rat, hamster, mouse and monkey (Hecht, 1998).

In most rodent tissues including lung and liver, NNAL is one of the major NNK metabolites formed *in vitro* (Hecht, 1998; Jalas *et al.*, 2005). Recent studies with rat lung suggest that the absolute configuration of NNAL may be important in NNK carcinogenicity. Based on relative differences in formation from NNK, accumulation in lung, metabolism to DNA reactive intermediates, detoxification and tumourigenicity, (*S*)-NNAL rather than (*R*)-NNAL may be the more important enantiomer in terms of contributing to NNK-induced lung tumour formation (Zimmerman *et al.*, 2004; Upadhyaya *et al.*, 2000).

NNK and NNAL detoxification by pyridine *N*-oxidation are relatively minor pathways in most rodents. In rat and mouse lung where NNK and NNAL *N*-oxidation occurs more extensively, NNK-*N*-oxide is the major *N*-oxide formed (Doerr-O'Rourke *et al.*, 1991; Smith *et al.*, 1992b; Peterson *et al.*, 1991a; Smith *et al.*, 1990).

Both NNK and NNAL α-carbon hydroxylation pathways are commonly observed in rodent pulmonary tissues (Hecht, 1998). In rodent lung, α-methyl hydroxylation exceeds α-methylene hydroxylation and overall α-hydroxylation activity is greater in the

lung than in the liver (Hecht, 1998). Moreover, it is thought that relatively high α-hydroxylation activity in rat lung may be one reason for the susceptibility of rat lung to NNK-induced carcinogenesis (Hecht, 1998).

NNK metabolism in different lung cell types has been investigated. In rat, hamster, rabbit, and A/J mouse, both non-ciliated bronchial epithelial (Clara) cells and alveolar type cells are major sites of NNK bioactivation (Belinsky *et al.*, 1989b; Belinsky *et al.*, 1991; Alaoui-Jamali *et al.*, 1990; Dahl *et al.*, 1990). However in rat, hamster and rabbit, Clara cells have higher NNK bioactivation activity than do other cell types (Belinsky *et al.*, 1991; Alaoui-Jamali *et al.*, 1990; Dahl *et al.*, 1990). Consistent with the inter-cellular differences in NNK activation are the differences in CYP content. In rodents and rabbits, Clara cells contain abundant smooth endoplasmic reticulum and are considered to be the predominant cellular site for CYPs (Devereux *et al.*, 1989; Massey *et al.*, 1987; Domin *et al.*, 1986). The alveolar type II cells possess lower CYP activity, followed by macrophages.

1.4.5 NNK Biotransformation in Human Lung

Human NNK metabolism has been assessed *in vivo* and *in vitro* in liver, lung, oral tissue, nasal mucosa, red blood cells, placenta, and pancreas using cultured tissue and cells, microsomes, cytosols and freshly isolated lung cells (Hecht, 1998; Smith *et al.*, 1999).

In human lung, NNAL is the major metabolite formed *in vitro* (Hecht, 1998; Smith *et al.*, 2003), with amounts of NNAL formed being greater than those produced in

rodent lung (Hecht, 1998). NNK is readily reduced to (*S*)-NNAL in human lung (Upadhyaya *et al.*, 2003) and while its accumulation has not be reported, this enantiomer is more slowly excreted in human urine than is (*R*)-NNAL (Carmella *et al.*, 1999), suggesting that similar to rat, (*S*)-NNAL rather than (*R*)-NNAL may be important in NNK carcinogenicity in humans. α-Hydroxylation of NNK and NNAL in human lung microsomes has been found to be much lower than in rodent lung microsomes (Hecht, 1998). In human lung, the major NNK metabolite (including NNAL derived metabolites) produced is keto acid, suggesting higher activity of the α-methylene hydroxylation pathway of NNK (Smith *et al.*, 2003).

Pyridine *N*-oxidation of NNK and NNAL has been much less characterized in human relative to rodents but has been observed in human lung microsomes and in isolated human lung cells (Smith *et al.*, 2003; Smith *et al.*, 1999).

While both Clara cells and alveolar type II cells are important sites for NNK metabolism in rodents, limited extrapolation of these results to humans can be made as there are significant species differences in the morphological and functional characteristics of lung cells (Plopper, 1983; Crapo *et al.*, 1982). Notably, Clara cells are rare in humans and are decreased in number by cigarette smoke (Lumsden *et al.*, 1984). As well, human Clara cells do not contain the large amounts of endoplasmic reticulum as found in rodent Clara cells (Smith *et al.*, 1979). Therefore it is likely that human Clara cells do not play as large of a role in xenobiotic biotransformation, suggesting that human alveolar type II cells, which contain higher levels of endoplasmic reticulum and have

relatively high CYP biotransformation activities are more likely involved (Devereux *et al.*, 1986; Anttila *et al.*, 1991).

To date only one study has examined NNK metabolism in isolated human lung cells. When comparing NNK α-hydroxylation and *N*-oxidation activities between alveolar type II cells and alveolar macrophages, no differences in the degree of total α-hydroxylation and total *N*-oxidation were found, although formation of the α-hydroxylation metabolite diol was significantly higher in type II cells (Smith *et al.*, 1999).

In all previous human studies, a large degree of interindividual difference in NNK metabolism has been observed (Smith *et al.*, 2003; Finckh *et al.*, 2001; Soldan *et al.*, 1999). Differences in the activities of NNK bioactivation and detoxification pathways is of importance as the balance between these pathways in human lung may contribute to individual susceptibility and thus may play an important role in lung cancer risk.

NNK is a potent pulmonary carcinogen in experimental animals and is thought to be responsible for the increasing incidence of human lung adenocarcinoma. Biotransformation of NNK is a critical determinant of NNK carcinogenicity and these processes, occurring predominantly in target organs and target cell types of NNK tumourigenesis, have been well characterized in experimental animals. However, knowledge of these processes in human lung is limited and while multiple enzymes and enzyme systems have been implicated, identification of the main enzymes involved in human pulmonary NNK metabolism remains to be elucidated.

1.5 DNA REPAIR AND NNK

1.5.1 DNA Repair Pathways

DNA repair systems have evolved to remove or overcome DNA lesions resulting from both endogenous and exogenous DNA-damaging agents. If these repair systems are overwhelmed or if defects in the pathways exist, mutations accumulate which can lead to the development of cancer. Six distinct DNA repair mechanisms have been identified in humans; mismatch repair, base excision repair (BER), nucleotide excision repair (NER), direct damage reversal, and homologous and non-homologous double-strand break repair (Hoeijmakers, 2001).

Mismatch repair removes base mismatches caused by spontaneous or induced base deamination, oxidation, methylation and replication errors. BER is responsible for the repair of DNA-damaged bases, mainly oxidized DNA bases, by a two-step process involving DNA glycosylases and endonucleases (Hoeijmakers, 2001). NER is a multi-step process involving more than 30 proteins that repairs bulky DNA adducts, intra-strand cross-links and large chemical adducts (Hoeijmakers, 2001). The direct damage reversal pathway is involved in the repair of O^6-alkylation lesions by the enzyme, O^6-alkylguanine-DNA alkyltransferases (AGT) which transfers the alkyl group from guanine to a cysteinyl residue at its active site (Christmann *et al.*, 2003). DNA double-stand break repair, including homologous recombination and non-homologous end-joining, is involved in the repair of DNA double-strand breaks which can induce chromosomal breaks and/or exchanges and cell death (Hoeijmakers, 2001). While each of these repair mechanisms removes DNA damage in a specific manner, there is some degree of overlap

between the types of DNA lesions repaired by each as well as some repair proteins that participate in each of the processes.

1.5.2 Nucleotide Excision Repair

NER is the most versatile DNA damage removal pathway since it deals with a wide range of structurally unrelated DNA helix-distorting lesions that interfere with base pairing and obstruct transcription and normal replication (Hoeijmakers, 2001).

NER consists of two distinct subpathways, global genome repair (GGR) and transcription-coupled repair (TCR) (Figure 1.4). GGR is a transcription-independent pathway and involves in the removal of lesions from non-transcribed regions of the genome and the non-transcribed strand of transcribed genes, while TCR is transcription-dependent and involves removal of RNA-polymerase-blocking lesions from transcribed strands of active genes (Christmann *et al.*, 2003). Notably, mutations due to damage in inactive genomic regions (repaired by GGR) are a better predictive marker for carcinogen-induced tumourigenesis than mutations in actively transcribed genes, suggesting that GGR, rather than TCR, plays a more important role in carcinogenesis (Balajee and Bohr, 2000). Both subpathways are mediated by the sequential assembly of repair proteins at the site of DNA damage and involve multiple steps including DNA lesion recognition, incision of the damaged DNA strand, excision of the damaged oligonucleotide, and resynthesis and ligation of DNA (Costa *et al.*, 2003). Although the two subpathways employ a common set of proteins for the incision/excision (including

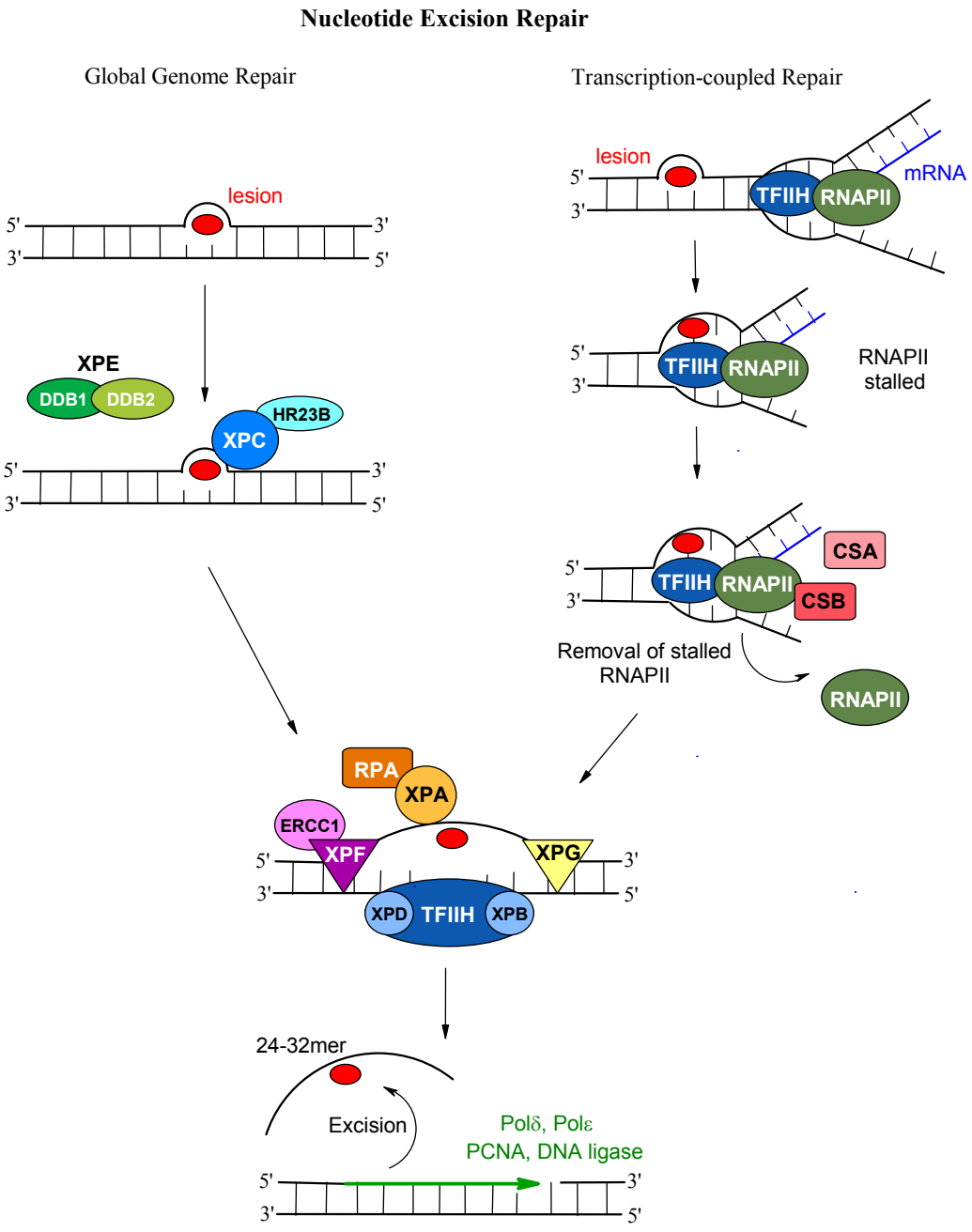

Figure 1.4 Simplified mechanism of two subpathways of NER, global genome repair and transcription-coupled repair (modified from Bedard and Massey, 2006). CSA and CSB, Cockayne's syndrome proteins A and B; DDB1 and DDB2, damaged DNA binding proteins; PCNA, proliferating cell nuclear antigen; Polδ and Polε, DNA polymerases; RNAPII, RNA polymerase II; RPA, replication protein A; XP, xeroderma pigmentosum.

TFIIH, XPG, XPA, RPA and XPF-ERCC1) and DNA resynthesis steps, they differ in their mode of damage recognition.

In GGR, DNA lesion recognition is based upon the structural distortion and/or chemical alteration of DNA. Damage recognition is the rate-limiting step (Balajee and Bohr, 2000) and the rate of DNA repair is strongly dependent on the helix distorting properties of the lesion and on chromatin structure and proteins bound to DNA (Hanawalt, 2002). DNA lesions that efficiently distort the DNA helix and significantly disrupt base pairing are removed more rapidly than less-distorting lesions. For instance, differences in DNA repair rates occur with structurally different types of UV-light induced lesions (Balajee and Bohr, 2000; Christmann *et al.*, 2003). UV-induced cyclobutane pyrimidine dimers are mildly helix-distorting and are repaired at a slower rate compared to the more structurally distorting (6-4) pyrimidine–pyrimidone UV photoproducts (Balajee and Bohr, 2000). The efficiency of DNA repair of these two lesions correlates with cytotoxicity and mutagenicity (Tang *et al.*, 1986). In addition to base pairing disruption, other parameters including DNA unwinding, bending and flexibility may all contribute to the amount of thermodynamic destabilization induced by DNA damage, and thermodynamic destabilization is the key parameter that determines the propensity of a lesion to be repaired by NER (Gillet and Scharer, 2006).

The xeroderma pigmentosum (XP) C protein is unique to GGR and is necessary for damage recognition and the early stages of repair protein recruitment. While it has been extensively debated as to which NER protein, XPC or XPA is responsible for the initial recognition of DNA damage, recently it was demonstrated that XPA requires the

presence of XPC in order for XPA to be recruited to the sites of DNA damage, and that XPC must arrive at the lesion before XPA (Volker *et al.*, 2001). XPC forms a tight complex with HR23B and although NER is functional with XPC alone, HR23B substantially stimulates the reaction (Gillet and Scharer, 2006) and stabilizes XPC by inhibiting its polyubiquitylation and degradation (Gillet and Scharer, 2006). The XPC-HR23B complex binds efficiently to single-stranded DNA and helix distortions irrespective of whether there is a lesion present (Gillet and Scharer, 2006). Therefore following recognition of distorted DNA by XPC-HR23B, a subsequent damaged verification step is needed.

An important factor involved in the UV damage recognition process and also unique to GGR is the damaged DNA binding protein (DDB). DDB is a heterodimer comprised of two polypeptides, DDB1 (p127) and DDB2 (p48) that each belong to the XPE complementation group (Christmann *et al.*, 2003). DDB is important in the recognition of mildly helix distorting lesions such cyclobutane pyrimidine dimers through its binding and subsequent recruitment of XPC-HR23B to lesions (Hwang *et al.*, 1999; Wakasugi *et al.*, 2002). Moreover DDB may be important *in vivo* in the identification of lesions that are poorly recognized by XPC-HR23B (Costa *et al.*, 2003).

In contrast to the damage recognition process involved in GGR, in TCR, the repair process is initiated when RNA polymerase II (RNAPII) is blocked at the DNA lesion site. Although proteins Cockayne's syndrome (CS) A and CSB are required in the early stages of TCR, their exact functions remain to be elucidated (Costa *et al.*, 2003). It is thought that RNAPII interacts directly with CSB but not CSA (Christmann *et al.*, 2003)

and this interaction releases the stalled RNAPII from the damaged DNA. Additionally, CSA- and CSB-dependent hyperphosphorylation (inactivation) and ubiquination of RNAPII at stalled transcription forks has been observed and these actions are believed to facilitate its displacement and degradation by proteolysis (Christmann *et al.*, 2003; Svejstrup, 2002).

After detection of the DNA lesion, both subpathways use the same repair factors to perform subsequent steps. Once XPC-HR23B has detected the lesion, the TFIIH complex is recruited by protein-protein interactions to the DNA lesion (Christmann *et al.*, 2003; Gillet and Scharer, 2006). Similarly, in TCR, CSB may play a role in TFIIH recruitment (Tantin, 1998) if TFIIH has been released from RNAPII following the initiation of transcription (Gillet and Scharer, 2006). TFIIH, which is also involved in transcription, is a ten-subunit complex composed of a core complex (XPB, XPD, p62, p52, p44, p32 and p8/TTDA) and a cyclin-dependent kinase-activating complex (CAK) (cdk7, MAT1 and cyclin H). The XPB and XPD proteins of the core complex are ATP-dependent helicases that are responsible for 3' to 5' and 5' to 3' DNA unwinding, respectively, at the site of DNA damage, which allows for entry of subsequent NER factors. The core complex but not the CAK complex supports the NER function of TFIIH, while both complexes participate in basal transcription (Coin *et al.*, 2006; Dubaele *et al.*, 2003). It is thought that TFIIH, in particular the helicases, contribute to damage verification following XPC binding as they would be stalled at the lesion upon unwinding of DNA (Gillet and Scharer, 2006). If a lesion is not encountered by TFIIH, it is not stalled and NER is not initiated.

Following the formation of an open complex as a result of TFIIH activity, RPA, XPA and XPG are recruited to form the preincision complex. RPA is required for both the dual incision and repair synthesis steps of NER (Gillet and Scharer, 2006). To prepare for incision, RPA binds to the non-damaged strand of the opened DNA, allowing for accurate positioning of the endonucleases XPG and ERCC1-XPF. In addition, RPA is considered a recognition factor as it recognizes the locally unwound single-stranded DNA regions induced by the DNA damage (Gillet and Scharer, 2006) and the specificity of RPA for damaged DNA is enhanced by XPA (Gillet and Scharer, 2006).

Although XPA participates in damage recognition, it functions after XPC binding to control the proper assembly of the NER preincision complex by probing the distorted DNA, thereby confirming the existence of the lesion indirectly (Gillet and Scharer, 2006). XPA has no catalytic activity but instead acts as a core structural factor, interacting with DNA and most proteins in the preincision complex including RPA, TFIIH and XPF-ERCC1 and XPC-HR23B (Li *et al.*, 1998; Gillet and Scharer, 2006). XPA also interacts with XPA1 and XPA2, which are involved in XPA nuclear translocation and CS protein interactions, respectively (Costa *et al.*, 2003).

XPG is believed to be recruited to the preincision complex by interacting with TFIIH (Gillet and Scharer, 2006). The main role of XPG is to perform the 3'-incision of the DNA lesion (Habraken *et al.*, 1994). However, it also stabilizes the preincision complex to allow XPF-ERCC1 to function (Gillet and Scharer, 2006). The XPF-ERCC1 complex is recruited to the preincision complex by XPA where it is responsible for the 5'-incision of damaged DNA (Gillet and Scharer, 2006; Sijbers *et al.*, 1996). XPF is always

complexed with ERCC1 because the two subunits are unstable without one another (Gillet and Scharer, 2006). Excision of the lesion by dual incisions occurs at defined positions flanking the DNA damage and results in removal of a damaged oligonucleotide 24-32 nucleotides long (Gillet and Scharer, 2006). Incisions by XPG and XPF-ERCC1 do not occur simultaneously but rather are independent with the 5' incision by XPF-ERCC1 following the 3' incision by XPG (Mu *et al.*, 1996).

After incision and excision of the damaged DNA strand, repair synthesis and ligation occurs. Repair synthesis requires the presence of multiple proteins that include the polymerase processivity factor PCNA, the clamp loader replication factor C (RFC), the PCNA-dependent DNA polymerases, DNA polymerase δ or ε and RPA (Gillet and Scharer, 2006). RPA is believed to be involved in coordinating incision and repair synthesis since it remains bound to the single-stranded DNA intermediate following dual incision and recruits PCNA and RFC to the incised site. Once DNA pol δ or ε fills in the DNA gap, it is sealed by DNA ligase I (Aboussekhra *et al.*, 1995; Araujo *et al.*, 2000; Mu *et al.*, 1995).

A hallmark NER deficiency disorder that is associated with defects in genes involved in NER is xeroderma pigmentosum (Sijbers *et al.*, 1996). Xeroderma pigmentosum is an autosomal recessive disorder characterized by extreme hypersensitivity to sunlight, due to deficient repair of UV-induced DNA damage and predisposition to UV-induced skin cancer. Seven complementation groups, XPA through XPG, corresponding to defects in the corresponding gene products of *XPA-XPG* genes,

have been described (Salles *et al.*, 1995a). Defects in these genes manifest themselves as defective GGR and in some cases defective GGR and TCR.

The NER process, specifically GGR but not TCR, has been reproduced in an *in vitro* biochemical assay (Wood *et al.*, 1988; Wood *et al.*, 1995), in which repair activity of cell-free nuclear protein extracts prepared from either cells or whole tissue is measured by the extent of DNA repair synthesis in damaged plasmid DNA. Moreover, it has been found that NER on plasmid DNA resembles genomic repair *in vivo* (Salles *et al.*, 1995a) and defective repair occurs in extracts from cells from individuals with xeroderma pigmentosum (Wood *et al.*, 1988; Aboussekhra *et al.*, 1995).

The NER process *in vivo* is more complex than explained thus far as DNA is not usually readily accessible but instead organized into chromatin structures called nucleosomes (DNA coiled around a histone core) and further condensed into chromosomes. Before NER can occur and DNA damage can be detected, chromatin must be remodeled and DNA must be temporarily displaced from the nucleosome structures. The process of chromatin remodeling occurs by two major mechanisms including post-translational modification of histones (i.e. histone acetylation) or ATP-dependent chromatin remodeling (Gillet and Scharer, 2006). While in the case of TCR, chromatin accessibility coincides with the transcription process (Rubbi and Milner, 2003), GGR seems to be dependent on the above processes for chromatin accessibility and efficient damage detection (Tijsterman *et al.*, 1999). Although the NER proteins XPA, RPA and XPC-HR23B have all been suggested as possible regulators of chromosome accessibility, their role in this process is unlikely for multiple reasons

including the fact that these proteins have a very low ability to bind to nucleosomal DNA lesions (Hara *et al.*, 2000; Yasuda *et al.*, 2005). P53, which is required for efficient GGR but not TCR, acts as a chromatin accessibility factor by mediating global chromatin relaxation through its effects on histone acetylation (Rubbi and Milner, 2003). It is thought that P53 induces chromatin decondensation prior to lesion detection, suggesting that P53 may play an active role in the regulation of GGR in response to DNA damage (Rubbi and Milner, 2003). In addition to its involvement in chromatin accessibility, P53 is involved in transcriptional induction and/or recruitment of repair proteins such as XPC, DDB2 (p48) (Wang *et al.*, 2003c), XPB (Chang *et al.*, 2008), TFIIH and RPA (Wang *et al.*, 2003c).

1.5.3 NNK-DNA Adduct Formation

Bioactivation of NNK produces both DNA methylating and DNA pyridyloxobutylating species, while bioactivation of NNAL results in the formation of DNA methylating and DNA pyridylhydroxybutylating species (Figure 1.5a).

NNK-induced DNA methylation pathways are very well characterized and it is known that methylation occurs at the 7- and O^6-positions of guanine and at the O^4-position of thymine (Hecht *et al.*, 1986; Belinsky *et al.*, 1986). Moreover all of these methyl-DNA adducts have been detected in DNA from tissue and cells of NNK-treated animals and 7-methylguanine (7-mG) has been detected in lung tissue from smokers (Hecht, 1998). The relative levels of the different methyl adducts vary both within and between tissues. Within all rodent tissues studied, levels 7-mG are the highest, followed

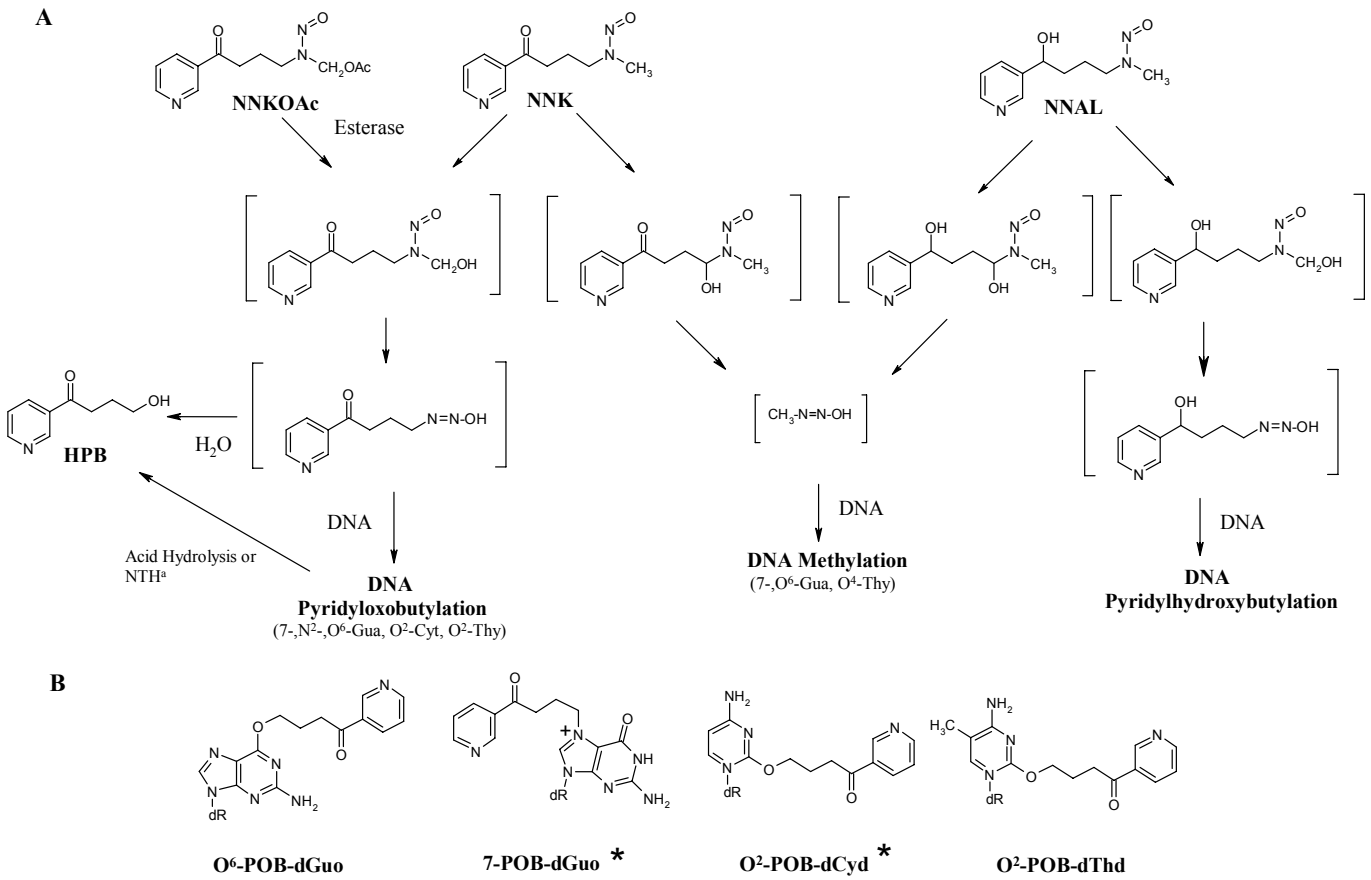

Figure 1.5 (A) Pathways for NNK, 4-(acetoxymethylnitrosamino)-1-(3-pyridyl)-1-butanone (NNKOAc) and NNAL bioactivation and DNA adduct formation. (B) Structures of the major pyridyloxobutyl (POB)-DNA adducts; * POB-DNA adducts that are unstable and release HPB. [a]Neutral thermal hydrolysis. HPB, 4-hydroxy-1-(3-pyridyl)-1-butanone.

by O^6-methylguanine (O^6-mG) and O^4-methylthymine (O^4-mT) (Table 1.2). When comparing individual methyl adduct levels between tissues, all adducts are found to a greater extent in lung, the principal target site of NNK carcinogenicity in rodents, versus liver (Hecht, 1998) (Table 1.2).

In contrast to methylation, NNK-induced DNA pyridyloxobutylation is not well characterized, largely due to the fact that the structures of the pyridyloxobutyl (POB) adducts were not identified until recently. The POB-DNA adducts that have been identified include 7-[4-(3-pyridyl)-4-oxobut-1-yl]-2'-deoxyguanosine (7-POB-dGuo), O^2-[4-(3-pyridyl)-4-oxobut-1-yl]-2'-deoxycytidine (O^2-POB-dCyd), O^2-[4-(3-pyridyl)-4-oxobut-1-yl]thymidine (O^2-POB-dThd) and O^6-[4-(3-pyridyl)-4-oxobut-1-yl]-2'-deoxyguanosine (O^6-POB-dGuo) (Wang *et al.*, 1997; Wang *et al.*, 2003b; Sturla *et al.*, 2005) (Figure 1.5b). Each of these adducts has been detected *in vitro* (Wang *et al.*, 2003b; Sturla *et al.*, 2005; Thomson *et al.*, 2004; Lao *et al.*, 2006) follwoing reaction of DNA with 4-(acetoxymethylnitrosamino)-1-(3-pyridyl)-1-butanone (NNKOAc), with 7-POB-dGuo being the major adduct formed, followed by O^6-POB-dGuo, O^2-POB-dThd and O^2-POB-dCyd (Lao *et al.*, 2006). NNKOAc is a chemically activated form of NNK that has been used experimentally because it forms POB adducts exclusively, analogous to those formed following reaction of bioactivated NNK with DNA (Figure 1.5a). Of the POB-DNA adducts identified, 7-POB-dGuo and O^2-POB-dCyd are unstable and release HPB under hydrolytic conditions. Also under these conditions, depurination and depyrimidation can occur by rupture of the glycosidic bond, producing 7-[4-(3-pyridyl)-4-oxobut-1-yl]guanine (7-POB-Gua) and O^2-[4-(3-pyridyl)-4-oxobut-1-yl]cytosine (O^2-

Table 1.2 Summary of *in vivo* DNA adduct formation in rodents following *in vivo* treatment with NNK

Adduct Levels in Different Tissues

Tissue	Adduct Levels [a]
F-344 rat lung	7-mG > HPB-releasing adducts > O^6-mG > O^4-mT POB adducts (high dose): 7-POB-Gua > O^2-POB-dThd > O^2-POB-Cyt > O^6-POB-dGuo POB adducts (low dose): O^2-POB-dThd > 7-POB-Gua > O^2-POB-Cyt > O^6-POB-dGuo
liver	7-mG > HPB-releasing adducts > O^6-mG > O^4-mT POB adducts (high dose): 7-POB-Gua > O^2-POB-dThd > O^2-POB-Cyt > O^6-POB-dGuo POB adducts (low dose): O^2-POB-dThd > 7-POB-Gua > O^2-POB-Cyt
A/J mouse lung	7-mG > O6-mG > HPB-releasing adducts > O^4-mT
liver	7-mG > O6-mG > HPB-releasing adducts

Tissue Levels of Different Adducts

Adducts	Tissue levels in F-344 rats	Tissue levels in A/J mouse
7-mG	Lung > liver[b]	Liver > lung
O^6-mG	Lung > liver[b]	Liver > lung
O^4-mT	Liver > lung	N/A
HPB-releasing adducts	Lung > liver[c]	Liver > lung
7-POB-Gua	Liver > lung	N/A
O^2-POB-dThd	Liver > lung	N/A
O^2-POB-Cyt	Liver > lung	N/A
O^6-POB-dGuo	Lung > liver[d]	N/A

[a] Relative adducts levels may be different depending on the cell type and time after NNK treatment; see refs (Hecht, 1998, Lao *et al.*, 2006, Lao *et al.*, 2007}

[b] At low doses of NNK; order reversed at high doses (low dose = 3-90 μg/kg/day, i.p.; high dose = 900-10,000 μg/kg/day, i.p.)

[c] At low doses of NNK; order reversed at high doses (low dose = 15-150 μg/kg/day, i.p.; high dose = 1,000-10,000 μg/kg/day, i.p.)

[d] At high doses of NNK; Not detected in liver at low doses (high dose = 5.2-20.7 mg/kg/day, i.p.; low dose = 470-1200 μg/kg/day in drinking water).

POB-Cyt) plus apurinic and apyrimidinic sites, respectively, on DNA(Wang *et al.*, 2003b; Sturla *et al.*, 2005).

Although the structures of POB-DNA adducts were identified only recently, the pyridyloxobutylation pathway was established over 20 years ago (Hecht *et al.*, 1988) and has been quantified indirectly by measuring HPB release as a reflection of DNA adduct levels (Hecht *et al.*, 1988; Foiles *et al.*, 1991). Using HPB release, the formation of POB-DNA adducts has been extensively studied in rodents (Nelson *et al.*, 1996; Peterson *et al.*, 1991b; Staretz *et al.*, 1997a) and POB-DNA adducts have been detected in lung tissue from smokers (Foiles *et al.*, 1991). At low doses of NNK in the rat, the levels of HPB released from lung DNA are higher than those from liver (Murphy *et al.*, 1990; Boysen *et al.*, 2003; Morse *et al.*, 1989; Peterson *et al.*, 1991b), whereas in the A/J mouse, levels of HPB-releasing adducts are higher in liver than in lung (Hecht, 1998) (Table 1.2).

Studies have compared the relative levels of each methyl adduct with the levels of HPB-releasing adducts (Hecht, 1998) (Table 1.2). In rat lung, HPB-releasing adducts are present at higher levels than O^6-mG and the highest levels of each of these adducts are found in Clara cells with lower levels in type II cells, and macrophages (Devereux *et al.*, 1988; Belinsky *et al.*, 1987; Staretz *et al.*, 1997a). Moreover, high levels of O^6-mG and HPB-releasing adducts in Clara cells and type II cells is consistent with these cells types being important sites for NNK tumourigenesis. Similarly, in A/J mouse lung, levels of O^6-mG are highest in both type II cells and Clara cells (Belinsky *et al.*, 1989a). In mouse liver, the order of relative adduct abundance is similar to that in lung (Table 1.2).

However, despite the higher levels of methyl and HPB-releasing adducts in liver relative to lung, NNK-induced tumour induction in liver is infrequent (Hecht, 1998).

Recently, individual POB-DNA adducts have been detected in rat and, consistent with previous studies, dose-dependent organ differences in adduct levels were observed (Lao *et al.*, 2006). At relatively high NNK doses, total POB adduct levels and levels of individual POB adducts, 7-POB-Gua, O^2-POB-dThd and O^2-POB-Cyt were higher in the non-susceptible organ, the liver, than in the susceptible organ, the lung, while the POB-DNA adduct, O^6-POB-dGuo was more abundant in lung (Lao *et al.*, 2006). In another study using a dosing protocol more representative of that experienced by smokers, rats that were treated chronically with a relatively low dose of NNK, had total POB-DNA adducts that were about 2 times higher in lung than in liver (Lao *et al.*, 2007). In contrast to results with high NNK doses where 7-POB-Gua was the major POB adduct formed *in vivo*, at low NNK doses O^2-POB-dThd was the major POB adduct formed in both lung and liver, suggesting possible differences in the relative formation and persistence of different POB adducts with different doses of NNK (Lao *et al.*, 2006; Lao *et al.*, 2007).

Similar to NNK-induced POB-DNA adducts, pyridylhydroxybutyl (PHB)-DNA adducts formed as a result of NNAL bioactivation have only recently been identified. Reactions of a chemically activated form of NNAL, 4-(acetoxymethylnitrosamino)-1-(3-pyridyl)-1-butanol (NNALOAc) with deoxyguanosine or DNA produces several PHB-DNA adducts, including 7-[1-hydroxy-1-(3-pyridyl)but-4-yl]dGuo (7-PHB-dGuo), O^6-[1-hydroxy-1-(3-pyridyl)but-4-yl]dGuo (O^6-PHB-dGuo), N^2-[1-hydroxy-1-(3-pyridyl)but-4-yl]dGuo(N^2-PHB-dGuo), O^2[4-(3-pyridyl)-4-hydroxybut-1-yl]dCyd (O^2-PHB-dCyd) and

O^2[4-(3-pyridyl)-4-hydroxybut-1-yl]dThd (O^2-PHB-dThd) (Upadhyaya *et al.*, 2003; Hecht *et al.*, 2004). Of these adducts, both 7-PHB-dGuo and O^2-PHB-dCyd can release 4-hydroxy-1-(3-pyridyl)-1-butanol and form 7-[1-hydroxy-1-(3-pyridyl)but-4-yl]Gua (7-PHB-Gua) and O^2[4-(3-pyridyl)-4-hydroxybut-1-yl]Cyt (O^2-PHB-Cyt), respectively (Lao *et al.*, 2007). The relative levels of these PHB adducts formed *in vitro* and their presence *in vivo* in animals or in human tissues has yet to be determined.

In addition to the production of DNA pyridyloxobutylating and pyridylhydroxybutylating species as a result of α-methyl hydroxylation of NNK and NNAL, respectively, this reaction also produces formaldehyde and until recently DNA damage induced by formaldehyde released from NNK and NNAL metabolism had not be investigated. Reactions of NNKOAc or NNALOAc with DNA resulted in the formation of the formaldehyde derived adducts, N^6-hydroxymethyl-deoxyadenosine (N^6-$HOCH_2$-dAdo), N^4-hydroxymethyl-deoxycytidine (N^4-$HOCH_2$-dCyd), N^2-hydroxymethyl-deoxyguanosine (N^2-$HOCH_2$-dGuo), and the cross-links di-(N^6-deoxyadenosyl)methane (dAdo-CH_2-dAdo), (N^6-deoxyadenosyl- N^2-deoxyguanosyl)methane (dAdo-CH_2-dGuo), and di-(N^2-deoxyguanosyl)methane (dGuo-CH_2-dGuo) (Cheng *et al.*, 2008). Moreover, although detected at significantly lower levels than POB-DNA adducts, N^6-HOCH2-dAdo and dAdo-CH2-dAdo have also been detected in lung and liver tissue from NNK treated rats (Wang *et al.*, 2007a), demonstrating that formation of formaldehyde from NNK and NNAL metabolism may also contribute to the DNA damaging effects induced by both carcinogens.

NNK can also affect the sugar phosphate backbone of DNA, producing alkylphosphotriesters. It is thought that the phosphate adducts (pyridyloxobutylphosphotriesters) in addition to AP sites produced by the pyridyloxobutylation pathways are mainly responsible for DNA single-strand breaks generated by NNK treatment (Lacoste *et al.*, 2006).

Even though the data supporting the importance of DNA methylation plays in NNK carcinogenesis are compelling, more recent identification and characterization of other NNK-induced DNA alkylation pathways is of importance as these pathways are known to also contribute to NNK tumourigenesis (DNA pyridyloxobutylation) or their roles remain to be elucidated (DNA pyridylhydroxybutylation or formaldehyde-derived DNA adducts). While there has been significant advancement in our knowledge surrounding pyridyloxobutylation pathways in recent years, these studies have been mainly limited to *in vitro* experiments and only recently has the importance of these adducts been investigated *in vivo* in rat. Investigation of the formation and relative abundance of individual POB-DNA adducts in target tissues of other animals models as well as in human lung would also be of interest. In addition, there is evidence that other POB-DNA adducts may be also be formed *in vitro* (Wang *et al.*, 2003b; Lacoste *et al.*, 2006) but whether these (relatively minor) adducts contribute to NNK carcinogenesis has yet to be determined.

1.5.4 Repair of NNK-induced DNA Damage

Although bioactivation and formation of DNA adducts are critical determinants of mutagenicity, the maintenance of genetic integrity depends on the ability to repair damaged DNA. The pathways involved in the repair of DNA methylation are well characterized. The primary mechanism by which O^6-mG adducts are removed from DNA is O^6-alkylguanine-DNA alkyltransferases (AGT) (Wang *et al.*, 1997). Similarly, AGT is also able to repair O^4-mT adducts but not as well as O^6-mG adducts (Zak *et al.*, 1994). Alternatively, AGT does not seem to be responsible for 7-mG repair, rather BER and/or NER are thought to be the primary repair pathways involved (Plosky *et al.*, 2002).

The processes involved in the repair of NNK-induced pyridyloxobutylation have not been well characterized. Interestingly, AGT, which normally transfers small alkyl groups from damaged bases to its active site, also effectively repairs O^6-POB-dGuo DNA adducts (Wang *et al.*, 1997). However, it is not known whether AGT also repairs other POB-DNA adducts. Although an earlier study suggested the involvement of base and/or nucleotide excision repair pathways in the repair of NNK- and NNKOAc-induced DNA damage, direct involvement of a particular repair pathway could not be established because findings were based on the use of DNA polymerase inhibitors which affect multiple repair pathways including NER, BER and mismatch repair (Cloutier and Castonguay, 1998).

Unexpectedly, A/J mouse AGT repairs O^6-POB-dGuo and O^6-mG with equal efficiency (Thomson *et al.*, 2003). However, lower levels of O^6-POB-dGuo than of O^6-mG occur in A/J mouse lung following *in vivo* treatment with NNK (Thomson *et al.*,

2003), suggesting that, in addition to differences in adduct formation, low levels of O^6-POB-dGuo may be due to efficient removal of O^6-POB-dGuo by repair pathways other than AGTs. Similarly, in rat lung, levels of methyl DNA adducts are greater than the levels of HPB-releasing adducts following NNK treatment, even though α-methyl hydroxylation (leading to pyridyloxobutylation) exceeds α-methylene hydroxylation (leading to methylation) in this tissue, demonstrating more efficient removal of POB-DNA damage, possibly by a repair pathway other than AGT (Hecht, 1998).

It is thought that differences in the levels of individual POB-DNA adducts at particular doses and between different doses of NNK, and differences in total POB-DNA adducts between organs may result at least partly from differences in DNA repair efficiencies of individual POB adducts. In fact, there are differences in persistence of individual POB adducts, suggesting differences in the relative DNA repair efficiencies among adducts. With chronic low dose NNK exposure, in rat lung and liver, the levels of O^2-POB-dThd increased over time, while the levels of 7-POB-Gua decreased over time (Lao *et al.*, 2007). This suggests that O^2-POB-dThd is repaired poorly *in vivo*, whereas 7-POB-dGuo is relatively efficiently removed (Lao *et al.*, 2007). In the same study, O^6-POB-dGuo was only detected in lung and not in the liver after 20 weeks of daily NNK treatment. This is consistent with the observation that AGT activity is about three times higher in rat liver than in rat lung (Peterson *et al.*, 2001). In contrast, in A/J mice, it appears that O^6-POB-dGuo may be repaired better in lung, since O^6-POB-dGuo is detected in liver and not lung after NNK treatment (Thomson *et al.*, 2003).

Although not necessarily considered a DNA repair mechanism, recently translesion synthesis has been demonstrated to be important for bypassing irrepairable DNA damage that has been encountered by DNA polymerases during replication. While this process can be non-mutagenic, some of the translesion polymerases that transit the lesions are error-prone and mutations can occur. The polymerases involved in bypassing NNK-induced DNA damage have yet to be fully characterized. The human replicative pol δ and members of the Y-family of human translesion polymerases, pol η, pol ι and pol κ, all copy past O^6-mG lesions, while only pol η is able to efficiently copy past O^6-POB-dGuo *in vitro* (Choi *et al.*, 2006). In addition, pol η and pol κ mainly insert the nucleotides dCTP and dTTP across from both lesions, while pol ι incorporates dTTP (Choi *et al.*, 2006). Incorporation of dTTP across from either lesion is consistent with G to A transition mutations that are observed as a result of both types of DNA damage (Hecht, 1998). Although to date relatively little is known about the role of translesion polymerases in NNK carcinogenesis, it is likely that this area of research will have an important impact on our understanding of the process involved in NNK mutagenicity.

While the pathways involved in the repair of NNK-induced DNA methylation have been well characterized, relatively little is known about the pathways involved in the repair of NNK-induced DNA pyridyloxobutylation. Moreover, it is likely that processes other than AGT-catalyzed POB repair are critical for the effective removal of these highly mutagenic DNA adducts. Thus, investigation of POB adduct repair may provide valuable insight into the processes that contribute to POB-DNA adduct persistence, mutagenicity and NNK carcinogenicity.

1.5.5 Post DNA-Binding Events

Mutations in codon 12 of the K-*ras* gene are commonly found in mouse lung tumours induced by NNK. The most common mutation is a GGT → GAT transition (~85%), with GGT → GTT, GTT → TGT and GGT → CGT transversions also occurring (Hecht, 1998). These types of mutations are consistent with roles for DNA methylation and DNA pyridyloxobutylation, since the formation and persistence of O^6-mG correlates with G to A transitions (Hecht, 1998) and POB adducts induce both G to T and G to A mutations (Kunkel, 1984; Pauly *et al.*, 2002). In addition, using K-*ras* sequence-specific double-stranded DNA and NNK activated intermediates, 7-mG has been shown to induce G to T transversions at the second G of codon 12 (Ziegel *et al.*, 2003). While K-*ras* mutations are common in mouse lung tumours, no mutations have been detected in the *Trp53* gene following NNK treatment, suggesting minimal involvement of point mutations in this gene in the development of NNK-induced lung adenocarcinoma (Matzinger *et al.*, 1995; Chen *et al.*, 1993). Similarly, in NNK-induced hamster lung adenocarcinoma, mutations in K-*ras* are present while mutations in *TP53* are absent (Oreffo *et al.*, 1993). In rat, there is neither evidence for *Trp53* nor K-*ras* mutations in lung tumours induced by NNK (Belinsky *et al.*, 1991), suggesting that other genes may be important in the initiation of lung carcinogenesis in this model.

Recently, the miscoding properties of individual POB-DNA adducts have begun to be determined. O^6-POB-dGuo is highly mutagenic in bacteria and human cells, causing primarily G to A transitions and a small number of G to T transversions (Pauly *et al.*, 2002). The O^2-POB-dThd lesion may be particularly important in NNK

carcinogenicity and mutagenicity in rats since it does not seem to be repaired well *in vivo* (Lao *et al.*, 2007). Although the miscoding properties of O^2-POB-dThd are currently not known, an analogue, O^2-ethyl-dThd is mutagenic, causing T to A transversions (Grevatt *et al.*, 1992; Bhanot *et al.*, 1992).

In addition to mutations that may arise from the DNA adducts, abasic sites produced as a result of the spontaneous depurination of 7-mG or 7-POB-dGuo or the depyrimidination of O^2-POB-dCyd (Ziegel *et al.*, 2003) can also cause mutations in mammalian cells (Gentil *et al.*, 1992; Avkin *et al.*, 2002).

Formation of both methyl and POB adducts has been proposed to be important in the induction of carcinogenicity by NNK since less potent nitrosamines only either methylate or pyridyloxobutylate DNA (Hecht, 1998). In A/J mice, the methylation pathway seems to be critical in NNK carcinogenesis (Staretz *et al.*, 1997a; Peterson and Hecht, 1991; Hecht *et al.*, 1990). In A/J mice, a strong correlation exists between the levels and persistence of O^6-mG and lung tumour multiplicity (Peterson and Hecht, 1991) and mutations in the K-*ras* oncogene from lung tumours of NNK treated mice are consistent with formation of O^6-mG (Ronai *et al.*, 1993). Although pyridyloxobutylating compounds alone are weak lung carcinogens in this model (Peterson and Hecht, 1991), they increase the tumourigenic activity of methylating agents by both generating adducts that contribute to the initiating activity of NNK and increasing the levels and persistence of O^6-mG through inhibition of AGT activity (Peterson *et al.*, 1993; Peterson *et al.*, 2001).

In contrast, both pyridyloxobutylation and methylation may be important in NNK-induced carcinogenesis in rat lung. Levels of HPB-releasing DNA adducts in alveolar type II cells correlate with lung tumour incidence over a range of doses of NNK (Belinsky *et al.*, 1990). Conversely, the levels of O^6-mG in Clara cells also correlate with lung tumour incidence, although some investigators believe that type II cells and not Clara cells are the cell of origin of the tumours (Belinsky *et al.*, 1990).

Although inter-species differences clearly exist in the relative importance of methylation and pyridyloxobutylation in the induction of lung carcinogenesis, the relative importance of these two pathways in human lung is not known. In human lung, 24-50% of adenocarcinomas contain mutations in codon 12 of K-*ras* and these mutations are more common in smokers than in nonsmokers (Hecht, 1999). Of these mutations, 80% are G to T transversions and 20% are G to A transitions. The high proportion of transversion mutations suggests a role for pyridyloxobutylation in NNK carcinogenesis in human lung (Hecht, 1999). However, since numerous DNA damaging compounds are present in tobacco smoke and many of these cause G to T tranversions, it is difficult to assign mutations to particular carcinogen adducts (Hecht, 1998).

In addition to mutations in K-*ras*, the vast majority of current or former smokers, in contrast to never-smokers, exhibit loss of heterozygosity (LOH) (the loss of one of the two alleles at one or more loci within the genome) at multiple allelic sites (e.g. p16, P53, chromosome 3p) in both normal and neoplastic bronchial epithelium (Mao *et al.*, 1997; Aoyagi *et al.*, 2001). In mouse lung, NNK increases the frequency of global LOH and specifically alters the frequency of LOH on chromosomes 12, possibly inactivating genes

that are associated with maintaining chromosomal stability (Herzog *et al.*, 2002; Herzog *et al.*, 2004). Although not found in NNK-induced lung tumours in rodents, both G to T and G to A mutations are prevalent in the *TP53* gene in human lung adenocarcinoma (Hainaut and Pfeifer, 2001). Moreover, in human genomic DNA treated with NNKOAc, several sites of POB damage are found in mutational hotspots of the *TP53* gene (Cloutier *et al.*, 2001).

Codon 12 of K-*ras* is a major mutational hotspot in smoking-induced lung adenocarcinoma and in NNK-induced mouse lung tumours and although not implicated in rodents, *TP53* mutations may be caused by NNK in smokers. Moreover, in addition to DNA methylation pathways, DNA pyridyloxobutylation pathways are also likely to play critical role in NNK-induced pulmonary carcinogenesis in both rodents and smokers.

1.6 RESEARCH HYPOTHESES AND OBJECTIVES

With lung cancer being the leading cause of cancer-related death in the world, further research is needed to understand the mechanisms of pulmonary carcinogenesis. NNK is a potent pulmonary carcinogen in experimental animals and may be an important contributor to the increasing incidence of human lung adenocarcinoma. Although biotransformation is a key process involved in NNK carcinogenicity, knowledge of this process and the main enzymes involved in human lung is limited. While evidence has implicated many different enzyme systems, it is thought that cytochromes P450 make the largest contribution to NNK metabolism in human lung. Moreover, recently it has been suggested that the CYP2A isozymes, CYP2A13 and CYP2A6 may play a particularly

large role in pulmonary NNK metabolism because of their relatively high catalytic efficiency for NNK bioactivation and their substantial expression in human lung. However, to date, the importance of CYP2A enzymes in human pulmonary NNK metabolism has yet to be determined. Determination of the major enzymes involved in NNK metabolism in human lung may provide further insight into the pathways involved in NNK metabolism, allow for the identification of individuals with high risk for cancer development based on relative NNK metabolism and enzyme activities and ultimately allow for the development of novel strategies aimed at the prevention of tobacco-induced lung cancer.

Although bioactivation and formation of DNA adducts are critical determinants of mutagenicity, the maintenance of genetic integrity depends on the ability to repair damaged DNA. While the pathways involved in the repair of NNK-induced DNA methylation have been well characterized, relatively little is known about the pathways involved in the repair of NNK-induced DNA pyridyloxobutylation. While there is evidence that AGT contributes to POB-DNA adduct repair, it is likely that other repair pathways are essential. Based on previous observations and because of the bulky and DNA helix distorting properties of POB adducts, it is likely that NER participates in their repair. Characterization of processes involved in POB adduct repair is of importance since POB adduct formation plays an important role in NNK-induced tumourigenicity in rodents (Staretz *et al.*, 1997a; Trushin *et al.*, 1994; Peterson *et al.*, 2001) and is likely to be involved in tobacco-induced cancers in smokers (Foiles *et al.*, 1991). Thus, elucidation of the processes involved in POB adduct repair may allow for the

identification of susceptible individuals based on their ability to repair POB DNA damage and also may allow for the development of therapeutic strategies aimed at increasing the activity and/or efficiency of specific DNA repair pathways.

Although NNK has been established as a highly selective inducer of lung carcinogenesis in animals and is believed to be a causal agent in the induction of human lung adenocarcinoma, the mechanisms underlying the susceptibility of lung to the carcinogenic effects of NNK have not been fully characterized. Although animal studies suggest that biotransformation may contribute to the organoselectivity of NNK, there is also evidence that DNA repair is a critical determinant. Determination of whether inter-organ differences in repair of NNK-induced DNA damage exists or whether NNK itself alters repair may allow for identification of a novel mechanism by which NNK is mediating its lung-specific effects and also may stimulate further research into the role of DNA repair in target-organ carcinogenesis by other carcinogens.

Based on preceding background information, three research hypotheses were formulated, and specific objectives were adopted to address the hypotheses.

Hypothesis 1: CYP2A enzymes contribute substantially to NNK biotransformation in human lung microsomes

Objective 1a: To determine NNK biotransformation in human whole peripheral lung microsomes from a sample of individuals.

Objective 1b: To determine if, among individuals, correlations exist between the degree of NNK metabolism and: i) CYP2A13 mRNA expression; ii) CYP2A6 mRNA expression; iii) CYP2A enzymatic activity using a CYP2A-selective substrate; and iv) CYP2A inhibition by an inhibitory antibody.

Objective 1c: To determine the influence of a CYP2A13 genetic polymorphism, Arg257Cys, on interindividual differences in NNK biotransformation.

Hypothesis 2: Nucleotide excision repair is involved in the repair of NNK-induced pyridyloxobutyl (POB) DNA adducts

Objective 2a: To prepare and characterize plasmid DNA with pyridyloxobutyl adducts which can be used as a substrate for the measurement of DNA repair activity.

Objective 2b: To determine the ability of nuclear protein extracts from normal cells and NER-deficient XPA and XPC cells to repair NNK-induced pyridyloxobutylation DNA damage.

Objective 2c: To determine the relative susceptibilities of normal and NER-deficient cell lines to NNKOAc cytotoxicity.

Hypothesis 3: *In vitro* DNA repair activity of NNK-induced pyridyloxobutylated DNA is greater in mouse liver extracts than in mouse lung extracts and *in vivo* treatment of mice with a tumourigenic dose of NNK inhibits repair of pyridyloxobutylated DNA by lung extracts and increases repair by liver extracts

Objective 3a: To compare *in vitro* DNA repair activities of nuclear protein extracts from mouse lung and liver towards NNK-induced POB DNA damage.

Objective 3b: To determine if *in vivo* treatment of mice with NNK alters DNA repair activity in mouse lung and liver.

Objective 3c: To determine if NNK affects incision activity and/or the levels and/or DNA-binding activity of NER incision proteins in mouse lung and liver.

CHAPTER 2
ANALYSIS OF CYP2A CONTRIBUTIONS TO METABOLISM OF 4-(METHYLNITROSAMINO)-1-(3-PYRIDYL)-1-BUTANONE IN HUMAN PERIPHERAL LUNG MICROSOMES

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2.1 INTRODUCTION

To induce carcinogenesis, NNK and its keto-reduced metabolite NNAL require metabolic activation via α-carbon hydroxylation (Figure 1.3). Hydroxylation of the α-methylene carbons of NNK and NNAL leads to the formation of DNA methylating species, while hydroxylation of the α-methyl carbons of NNK and NNAL results in the formation of DNA pyridyloxobutylating and pyridylhydroxybutylating species, respectively. α-Carbon hydroxylation of NNK and NNAL also results in the formation of four endpoint metabolites, keto acid, keto alcohol, hydroxy acid and diol. These endpoint metabolites are used in assessing the degree of NNK bioactivation, since their formation is indicative of the formation of the DNA-reactive metabolites. The detoxification of NNK and NNAL occurs mainly through pyridine *N*-oxidation, which results in the formation of excretable *N*-oxides (Hecht, 1998).

Cytochromes P450, specifically CYP2A6/2A13, CYP2B6, CYP3A4/3A5 and CYP2E1 (Smith *et al.*, 1992a; Smith *et al.*, 1995; Hecht, 1998; Smith *et al.*, 1999; Smith *et al.*, 2003), and prostaglandin H synthase (Smith *et al.*, 1995; Smith *et al.*, 1999; Smith *et al.*, 2003) but not lipoxygenases (Bedard *et al.*, 2002) have been implicated in human

pulmonary NNK metabolism. Of particular interest are the CYP2A isozymes CYP2A13 and CYP2A6. CYP2A13 is expressed predominantly in the human respiratory tract including the nasal mucosa, trachea and peripheral lung (Su *et al.*, 2000), and heterologously-expressed CYP2A13 exhibits higher catalytic activity for NNK activation than do other CYP isoforms examined (Su *et al.*, 2000; Smith *et al.*, 1992a; Patten *et al.*, 1996). A functional polymorphism resulting from a C/T transition in exon 5 of the *CYP2A13* gene that leads to an Arg/Cys amino acid substitution at residue 257, significantly reduces the enzyme's activity towards several different substrates, including NNK (Zhang *et al.*, 2002). A potential protective effect against NNK-induced carcinogenesis for individuals possessing this variant allele is supported by an epidemiological study which found that individuals with variant CYP2A13 genotype (CT and TT) had a reduced risk of lung AC compared to individuals with wildtype (CC) genotype (Wang *et al.*, 2003a). The other CYP2A isozyme, CYP2A6, is the main CYP2A isoform in liver (Su *et al.*, 2000) but is also present in human nasal mucosa, trachea and lung (Su *et al.*, 2000). CYP2A6 is a major catalyst of nicotine and coumarin metabolism (Messina *et al.*, 1997; Fernandez-Salguero and Gonzalez, 1995) and is thought to be one of the major CYP isoforms responsible for NNK activation (Yamazaki *et al.*, 1992; Smith *et al.*, 2003). The role of CYP2A6 in lung cancer is not clear as studies that have examined the relationship between *CYP2A6* polymorphisms and lung cancer risk have been conflicting. In one study CYP2A6 deficiency has been correlated with reduced lung cancer risk (Miyamoto *et al.*, 1999) while another study showed no relationship (Wang *et al.*, 2003a). Because of their established involvement in NNK

bioactivation and their expression in human lung, it can be suggested that CYP2A13 and CYP2A6 may contribute largely to human pulmonary NNK metabolism.

In the present study, lung tissue from a relatively large sample size of individuals (n=29) was used to assess NNK biotransformation among individuals, and specifically the importance of both CYP2A13 and CYP2A6 in these pathways. In addition, the influence of the CYP2A13 Arg257Cys genetic polymorphism on NNK metabolism was assessed.

2.2 MATERIALS AND METHODS

2.2.1 Chemicals

Chemicals were obtained as follows: [5-^{3}H]NNK (2.4-11.0 Ci/mmol; >98% pure) from Chemsyn Science Laboratories (Lenexa, KS) and Moravek Biochemicals (Brea, CA); NNK, NNAL, 4-(methylnitrosamino)-1-(3-pyridyl-N-oxide)-1-butanone (NNK-*N*-oxide), 4-(methylnitrosamino)-1-(3-pyridyl-N-oxide)-1-butanol (NNAL-*N*-oxide), 4-hydroxy-1-(3-pyridyl)-1-butanone (keto alcohol), 1-(3-pyridyl)-1,4-butane diol (diol), 1-(3-pyridyl)-1-butanone-4-carboxylic acid (keto acid), 1-(3-pyridyl)-1-butanol-4-carboxylic acid (hydroxy acid), from Toronto Research Chemicals (North York, ON); Uniscint BD radioflow scintillation cocktail from National Diagnostics (Atlanta, GA); glucose-6-phosphate dehydrogenase, coumarin, 7-hydroxycoumarin and 7-hydroxy-4-methylcoumarin from Sigma-Aldrich Co. (St. Louis, MO). All other chemicals were reagent grade and were obtained from common commercial suppliers.

2.2.2 Tissue Procurement

Sections of peripheral human lung tissue devoid of visible tumors were obtained from patients undergoing clinically indicated lobectomy at Kingston General Hospital. Tissue specimens were cut into 1.5-cm^3 pieces, wrapped in aluminum foil, frozen in liquid N_2, and stored at -80°C until microsome preparation (Smith *et al.*, 2003). Histological analysis was performed on tissues to confirm the absence of microscopic tumors. Data regarding surgical diagnosis, gender, smoking history, potential occupational carcinogen exposure and drug treatments for the month prior to surgery were collected to identify possible confounders including the possible inductive/inhibitory effects of certain drug treatments on biotransformation enzymes. Patients were classified as former smokers if smoking termination was reported to be >2 months before surgery (McLemore *et al.*, 1990).

2.2.3 Preparation of Human Lung Microsomes

Human whole peripheral lung microsomes were prepared as described (Smith *et al.*, 2003). Briefly, tissue specimens were thawed on ice for 15 min. They were then rinsed, chopped, and homogenized in 0.1 M potassium phosphate buffer containing 1.15 % KCl (pH 7.4) using a Polytron homogenizer, and microsomes were prepared by differential centrifugation (Donnelly *et al.*, 1996). Protein concentration was determined by the method of Lowry *et al.*, (1951) using bovine serum albumin as a standard.

2.2.4 Incubations with NNK

Before use, the purity of [5-^{3}H]NNK was assessed, since high purity (>98%) was needed to ensure that impurities would not interfere with quantification of metabolites. If purity was <98%, [5-^{3}H]NNK was purified by HPLC as described (Smith *et al.*, 1999). Incubation mixtures were prepared as described (Smith *et al.*, 2003), using 4.2 μM [5-^{3}H]NNK and 1.0 mg microsomal protein in a total volume of 1.0 ml. In addition to samples that contained the complete incubation mixture, samples without the NADPH-generating system and samples bubbled with carbon monoxide (CO) were also prepared to assess the overall contribution of P450s. All metabolite analyses were performed in duplicate. NNK metabolite formation by human lung microsomes has been demonstrated to be linear for at least 30 min (Smith *et al.*, 2003).

2.2.5 Assessment of NNK Biotransformation

NNK metabolites were quantified by reverse-phase gradient HPLC with radiometric detection (Smith *et al.*, 2003). For immunoinhibition, 1.0 mg of microsomal protein and 200 μg of anti-CYP2A6/13 (BD Gentest, Woburn, MA) were incubated on ice for 20 min prior to use in incubations, as recommended by the supplier. For each metabolite, the amount produced was expressed as a percentage of the total radioactivity recovered from [5-^{3}H]NNK plus metabolites per milligram of protein per minute, to account for differences in recovery of [5-^{3}H]NNK between incubates. Metabolite peaks were quantified only if they were at least twice background radioactivity levels.

2.2.6 CYP2A mRNA Expression

Total RNA was isolated from human lung tissue (n=28) using the Qiagen RNeasy Mini Kit, with an additional on-column DNase treatment step in accordance with the manufacturer's instructions (Qiagen, Valencia, CA). The quality of the RNA samples was determined by electrophoretic analysis of 3 μg of RNA on a denaturing gel. Ethidium bromide staining of the gel detected distinct 28S and 18S rRNA bands with an intensity ratio of 28S:18S of at least 1.5. The UV absorbance ratio (260 nm/280 nm) ranged from 1.8 to 2.1 for all RNA samples. cDNA was synthesized from 5μg of total RNA in a reaction volume of 50 μl using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). For quantitative real-time PCR, 2μl aliquots of cDNA were amplified using CYP2A13 and CYP2A6 TaqMan primer and probes sets (Applied Biosystems, Foster City, CA; Assay ID Hs00426372_m1 and Hs00868409_s1, respectively) according to the manufacturer's recommendations. Amplification, detection and analysis were performed using Smart Cycler II instrumentation and software (Cepheid, Sunnyvale, CA). CYP2A13 and CYP2A6 gene expression was normalized using GAPDH as an endogenous reference. For mRNA quantitiation, the comparative C_T method was used since the relative PCR efficiencies for target and reference amplification were approximately equal. PCR product specificity was verified by the presence of a single PCR product after performing agarose gel electrophoresis with ethidium bromide staining. Due to limitations in tissue availability both CYP2A13 and CYP2A6 gene expression could not be assessed for all individuals.

2.2.7 Coumarin 7-Hydroxylase Activity

Currently, there are no CYP2A13 or CYP2A6 isozyme-specific substrates available. Since CYP2A13 and CYP2A6 both metabolize coumarin at the 7-position, coumarin 7-hydroxylation is often used as a probe activity for the CYP2A enzymes (von Weymarn *et al.*, 2005). 7-Hydroxycoumarin formation was assessed in isolated human lung microsomes (n=28) and in cDNA-expressed human CYP2A6 microsomes (BD Biosciences, Woburn, MA) as a positive control, according to the manufacturer's protocol, but with some modifications. Reaction mixtures containing 0.2 mM coumarin in a 250 µl total reaction volume were pre-incubated at 37°C for 5 min and then incubated for an additional 25 min after addition of 0.3 mg of microsomal protein. Levels of 7-hydroxycoumarin and the internal standard, 7-hydroxy-4-methylcoumarin were assessed using a Shimadzu HPLC system (Mandel Scientific, Guelph, ON) with RF-10Axl fluorescence detector and CLASS-VP (v. 7.2.1 SP1) software as described (www.cypex.co.uk/) with some modifications. The mobile phase consisted of 75/25 (v/v) 0.05% orthophosphoric acid/acetonitrile, delivered isocratically at 1.0 ml/min through a Supelcosil 5 micron LC-18 (15 cm by 4.6 mm) column (Sigma-Aldrich, St. Louis, MO) at room temperature. Fluorescence was determined with excitation at 324 nm and emission at 458 nm. Levels of 7-hydroxycoumarin formation were quantified by subtracting the fluorescence of boiled microsome samples and comparing to a standard curve of 7-hydroxycoumarin to 7-hydroxy-4-methylcoumarin peak area ratios as a function of 7-hydroxycoumarin concentration. 7-hydroxycoumarin formation was linear up to 1 mg of microsomal protein and for 30 min.

2.2.8 *CYP2A13* Ag257Cys Polymorphism Analysis

Genomic DNA was isolated from peripheral lung tissue by protease digestion followed by standard phenol:chloroform extraction and ethanol precipitation (Devereux *et al.*, 1993). Genotypes (n=84) for *CYP2A13* at the C3375T (Arg257Cys) site were assessed by PCR-RFLP analysis (Wang *et al.*, 2003a) with some modifications. Reaction conditions were as follows: 15 min at 95°C, 13 cycles of 30 s at 94°C, 30 s at 63°C (step-down 0.5°C/cycle) and 50 s at 72°C, followed by 20 cycles of 30 s at 94°C, 30 s at 57.5°C and 50 s at 72°C and a final elongation step of 5 min at 72°C. The PCR reaction resulted in a 375-bp product. Ten µl of PCR product were digested with 2.9 units of *Hha*I (New England Biolabs, Inc., Beverly, MA) and restriction products were separated on a 3% agarose gel. The wild-type C allele had a *Hha*I restriction site that resulted in two bands (217 and 158 bp) and the variant T allele resulted in the elimination of the restriction site, producing a single 375-bp band.

2.2.9 Data Analysis

For microsomal NNK biotransformation, individual metabolite values represent the mean of duplicate incubations. When grouped, microsomal data are presented as means ± standard deviations (SD). Statistically significant differences in grouped microsomal data were determined by a Student's *t* test and a Student's *t* test with Welch's correction if heterogeneity of variance was present. All correlation analyses were performed using Pearson Product Moment correlation analysis (GraphPad Prism 4 Software). $P<0.05$ was considered statistically significant in all cases. To determine if NNK bioactivation and detoxification group data were normally distributed, an

Anderson-Darling Normality test was used. Group data with $P<0.05$ were considered to have a non-normal distribution.

2.3 RESULTS

2.3.1 Patient Demographics

Microsomes were prepared from sections of peripheral lung obtained from 30 human subjects (17 males and 13 females) from eastern Ontario, aged 62.6 ± 10.5 years (Table 2.1). Due to limitations in tissue availability, microsomes from all 30 subjects could not be used in all analyses. Based on reported smoking histories, 16 individuals were current smokers, 12 were former smokers and information for two individuals was not available.

2.3.2 NNK Biotransformation in Lung Microsomes

NNAL was the major metabolite produced from NNK, with formation ranging from 0.280 to 0.900%/mg protein/min (representing ~ 8 to 27 pmol NNAL/mg protein/min). Total bioactivation, represented by the sum of the four α-carbon hydroxylation endpoint metabolites, ranged from $< 3.33 \times 10^{-4}$ to 7.50×10^{-2} % total α-hydroxylation/mg protein/min (representing ~ 0.01 to 2.3 pmol α-hydroxylation/mg protein/min). Total detoxification by *N*-oxidation, represented by the sum of the two *N*-oxides, ranged from $< 3.33 \times 10^{-4}$ to 1.33×10^{-3} % total *N*-oxidation/mg protein/min (representing ~ 0.01 to 0.04 pmol *N*-oxidation/mg protein/min). In all metabolism analyses, individuals with metabolism below the lower limit of detection were assigned a value of 0 % total metabolism/mg protein/min. Normality tests of bioactivation and

Table 2.1 Patient Demographics

Patient Code[a]	Age	Sex	Smoking History	Diagnosis Leading to Surgery	Drug Tx 1 Month Prior/Possible Occupational Exposure to Carcinogens
1IM	66	M	Current, 40 pckyrs	Squamous cell carcinoma	diltiazem HCL, salbutamol, budesonide, ranitidine, ASA
2LM	41	F	Current, 20 pckyrs	Adenocarcinoma	Sotalol HCL, cilazapril, estrogen, omeprazole Mg
1LM	61	F	Current, 60 pckyrs	Adenocarcinoma	formoteral, budesonide, nizatidine
6JM	46	M	Current, 31 pckyrs	Squamous cell carcinoma	bupropion, codeine syrup
1KM	58	M	Current, 46 pckyrs	Adenocarcinoma	chlorodiazepoxide HCL, NSAID, carbamazapine, chlorodiazapam, flotatenine, lamotrigine
4JM	55	M	Current, 40-60 pckyrs	Non-small cell carcinoma	ASA, 3 glasses wine/day
2JM	60	F	Current, 40 pckyrs	Adenocarcinoma	conjugated estrogens, oxybutynin chloride, medroxyprogesterone, acetaminophen
5KM	68	M	Current, 55 pckyrs	Squamous cell carcinoma	acetaminophen, meloxicam
6KM	77	M	Former, 50 pckyrs	Squamous Carcinoma	metformin, ramipril, atenolol
4KM	69	M	Former, 35 pckyrs	Adenocarcinoma	atenolol, spironlactone/ Silica exposure
3KM	71	F	Former, 35 pckyrs	Adenocarcinoma, non-small cell carcinoma	pantoprazole, lexothyroxine sodium, clonidine, amitriptyline, lorazepam, lovastatin, salbutamol
8KM	55	F	Former, 15 pckyrs	Adenocarcinoma	conjugated estrogens, paroxetine HCL, hydrochlorothiazide, verapamil, montelukast sodium, fluticasone proprionate, naratriptan, ASA
7KM	56	F	Former, 28 pckyrs	Non-small cell carcinoma	sertraline, conjugated estrogens, ranitidine, meloxicam
1JM	55	F	Current, N/A	Adenocarcinoma	terbutaline sulfate, salbutamol, ipratropium bromide, steroids, glyburide, metformin, cisapride monohydrate, lansoprazole, fluoxitine HCL, budesonide, trazodone
3LM	61	M	Current, 20-30 pckyrs	Squamous Cell Carcinoma	Exposure to insulation
9KM	71	M	Former, 50 pckyrs	Non-small cell adenocarcinoma	None
5LM	73	F	Former, 4 pckyrs	Non-small cell adenocarcinoma	Amitriptyline

8JM	42	F	Current, 24 pckyrs	Adenocarcinoma	
7JM	57	F	Former, 40 pckyrs	Adenocarcinoma	Teacher
9JM	58	M	Current, 69 pckyrs	Adenocarcinoma	chlorodiazepoxide HCL, carbamazapine, chlorodiazapam, NSAID, ASA, flotatenine, lamotrigine
3JM	45	M	Current, 49.5 pckyrs	Adenocarcinoma	diazepam, fluvoxamine, warfarin
6LM	78	M	Current, 60 pckyrs	Non-small cell carcinoma/Adeno-squamous carcinoma	None
9LM	78	M	Former, N/A	Squamous Carcinoma	None
7LM	64	M	Former, 52.5 pckyrs	Non-small cell carcinoma	budesonide/formoterol fumarate, salbutamol, simvastatin, fluticasone
8LM	59	M	Current, N/A	Squamous cell carcinoma	ASA, pravastatin, glyceryl trinitrate
1MM	54	F	N/A, 39 pckyrs	Adenocarcinoma	None
2MM	65	M	Current, 40 pckyrs	Adenocarcinoma	alcohol, omeprazole Mg, ramipril
3MM	72	F	Former, 30 pckyrs	Adenocarcinoma	nifedipine, telmisartan, venlafaxine, atorvastatin, ASA
5JM	69	M	Former, 48 pckyrs	Squamous cell carcinoma	diazepam, ranitidine
6HM	68	F	Former, 40 pckyrs	Adenocarcinoma	levo thyroxine, oxazepam, multivitamin

Abbreviations: M, Male; F, Female; pckyrs, (Pack years; number of packs of cigarettes smoked per day times the number of years); N/A, information not available.
[a] Microsomes assigned codes for patient confidentiality

detoxification group data revealed that each group was not normally distributed (P<0.05). Subsequently, based on total bioactivation, subjects could be classified as either high (17 subjects) or low (12 subjects) bioactivators, with significantly different mean total α-hydroxylation activities ((5.26 ± 1.23)x10^{-2} and (6.49 ± 5.90)x10^{-3} % total α-hydroxylation/mg protein/min, respectively, n=29, P< 0.05) (Figure 2.1a). Similarly, based on total detoxification, subjects could be grouped into high (9 subjects) and low (20 subjects) categories, with significantly different mean total *N*-oxidation activities ((2.03 ± 1.65)x10^{-3} and (2.50 ± 3.04)x10^{-4} % total *N*-oxidation/mg protein/min, respectively, n=29, P<0.05) (Figure 2.1b). No correlation between NNK bioactivation and detoxification activities for individuals was found (P>0.05). Also, no correlation was found between the degree of NNK bioactivation or detoxification and patients' age, gender or smoking status (P>0.05). Smoking status was compared among high and low bioactivation groups and it was found that 71% of high bioactivators and 36% of low bioactivators were current smokers. Potential effects of drug treatments on NNK metabolism were assessed and no observable differences were found in the NNK metabolite profiles of individuals taking drugs that may alter the activities of relevant CYPs, as compared to those of other subjects. NNAL formation was not significantly different between the high and the low bioactivation or detoxification groups (P>0.05).

Formation of the NNK-derived α-hydroxylation metabolites keto acid and keto alcohol was significantly higher than formation of the NNAL-derived hydroxy acid and diol, in both the high and low bioactivation groups (P<0.05) (Table 2.2). When comparing metabolite formation between groups, production of hydroxy acid, keto acid

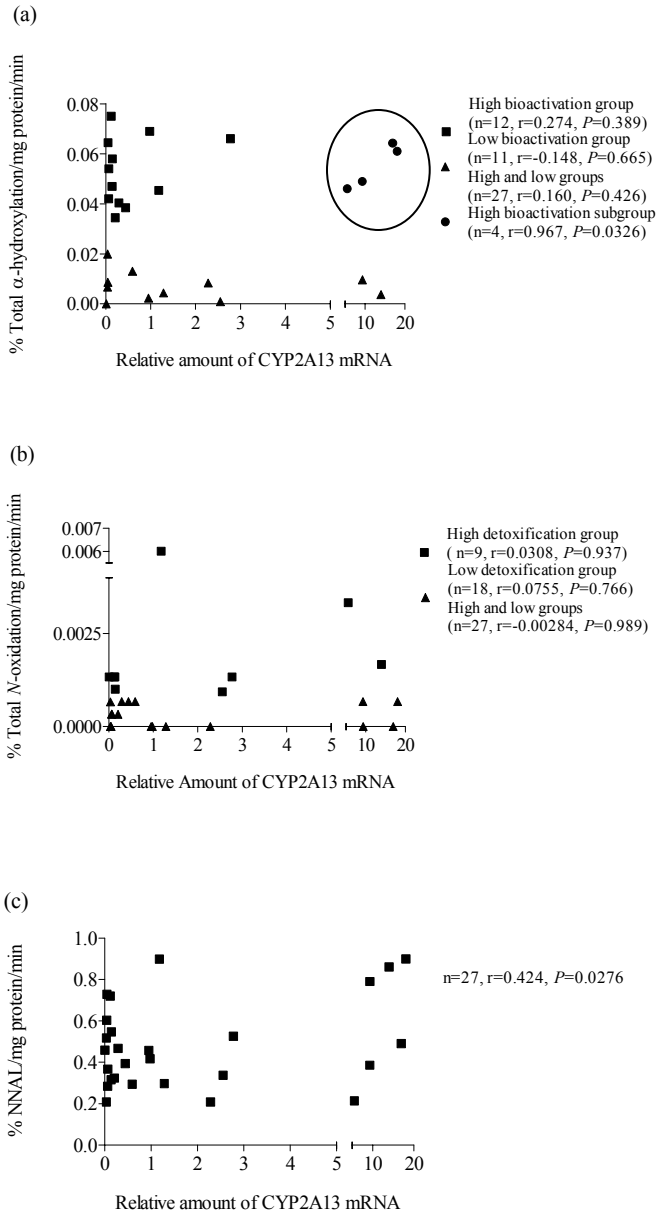

Figure 2.1 Correlation between the relative amount of CYP2A13 mRNA in human lung and the degree of NNK: (a) total α-hydroxylation (sum of four α-hydroxylation endpoint metabolites); (b) total *N*-oxidation (sum of two pyridine-*N*-oxidation metabolites); and (c) NNAL formation for each individual (n=27, Pearson's correlation). Levels of mRNA were determined by quantitative real-time RT-PCR and assessed using the comparative C_T method of relative quantitation, and are reported as values relative to patient 4JM. The high bioactivation subgroup (in part a) is enclosed within circle.

Table 2.2 Production of the four α-hydroxylation endpoint metabolites among high and low bioactivation groups

	% Metabolite formation/mg protein/min					
	HA	Diol	HA + Diol	KAC	KAL	KAC + KAL
High bioactivation group	6.08 ± 4.45 *[a]	16.3 ± 43.3	22.4 ± 43.5†	350 ± 109 *	157 ± 123 *	192 ± 164*
Low bioactivation group	1.39 ± 2.23	1.39 ± 2.23	2.78 ± 3.15†	56 ± 54.2	6.11 ± 19.2	62.1 ± 57.5

[a] Data are presented as the mean ± SD metabolite formation values for each of the four metabolites in each group x 10^{-4}.
* Significantly different from low bioactivation group, Student's *t* test with Welch's correction.
† Total NNAL-derived metabolites (hydroxy acid plus diol) are significantly different from total NNK-derived metabolites (keto acid plus keto alcohol) in both bioactivation groups, Student's *t* test with Welch's correction.
Abbreviations: HA, hydroxy acid; KAC, keto acid; KAL, keto alcohol.

and keto alcohol was significantly higher (~4 times, ~6 times and ~26 times, respectively) in the high compared to the low bioactivation group, while formation of diol, which was highly variable between individuals, was not significantly different between groups (P>0.05). Formation of total NNK-derived metabolites (i.e. keto acid plus keto alcohol) but not total NNAL-derived metabolites (i.e. hydroxy acid plus diol) was significantly different between the high and low bioactivaton groups (P<0.05).

Removal of the NADPH-generating system decreased total α-hydroxylation in tissues from the majority of subjects, by 3.6 to 100 % (median decrease =79.6%, n=21), but had increased or no apparent effect in microsomes from three subjects. Treatment with CO decreased total α-hydroxylation by 54.4 to 100 % (median decrease =66.7%) in microsomes from nine subjects and increased bioactivation by 2.12 to 140 % (median increase =34%) in microsomes from 13. Similarly, removal of the NADPH generating system decreased total *N*-oxidation by 25.0 to 100 % (median decrease =100%) in 13 subjects' microsomes, increased *N*-oxidation by 13.3 to 66.7 % (median increase =59.9%) in three, and had no effect in eight. CO decreased total *N*-oxidation by 25.1 to 100 % (median decrease =100%) in 14 subjects' microsomes, increased *N*-oxidation by 1.01 to 300 % (median increase =41.7%) in six, and had no apparent effect on NNK and NNAL *N*-oxidation in microsomes from four subjects. CO treatment eliminated NNAL formation in microsomes from two subjects and increased it by 3.77 to 329 % (median increase =165%) in 22 subjects. In contrast, removal of the NADPH-generating system completely eliminated NNAL formation in microsomes from all subjects (n=24).

2.3.3 CYP2A13 mRNA Expression and NNK Biotransformation

Regardless of whether high and low bioactivators and detoxifiers were considered separately or pooled, there was no significant correlation between CYP2A13 mRNA levels and the degree of total NNK bioactivation (Figure 2.1a) or detoxification (Figure 2.1b). Also, no significant associations were found between CYP2A13 mRNA levels and formation of individual α-hydroxylation metabolites. However, examination of the CYP2A13 mRNA and total α-hydroxylation scatter plot revealed the existence of a subgroup (n=4) with both high levels of CYP2A13 mRNA and a high degree of total α-hydroxylation, for whom CYP2A13 levels positively correlated with the degree of NNK bioactivation (r=0.967, $P<0.05$). Analysis of the two *N*-oxides (detoxification products) independently revealed no significant correlations between CYP2A13 mRNA expression and levels of NNAL-*N*-oxide (r=0.294, $P>0.05$) or NNK-*N*-oxide (r=-0.022, $P>0.05$). However, when subjects that had no detectable NNAL-*N*-oxide formation were excluded, a significant association was found between CYP2A13 mRNA expression and levels of NNAL-*N*-oxide (n=5, r=0.925, $P<0.05$). Of these five individuals, three were high bioactivators. A statistically significant correlation occurred between the extent of NNAL formation and CYP2A13 mRNA expression ($P<0.05$) (Figure 2.1c). However, when individuals (n=3) with relatively high levels of CYP2A13 mRNA and high levels of NNAL formation were excluded from the analysis, this correlation was no longer significant (r=-0.0658, $P>0.05$).

2.3.4 CYP2A6 mRNA Expression and NNK Biotransformation

When high and low bioactivators and detoxifiers were considered separately or pooled, there was no significant correlation between CYP2A6 mRNA levels and the degree of total NNK bioactivation (Figure 2.2a), detoxification (Figure 2.2b) or NNAL formation (Figure 2.2c). Similarly, no correlation was found between levels of CYP2A13 and CYP2A6 mRNA among individuals (n=24, r=0.114, P>0.05).

2.3.5 Coumarin 7-Hydroxylase Activity and NNK Biotransformation

Regardless of whether high and low bioactivators and detoxifiers were considered separately or pooled, there was no significant correlation between coumarin 7-hydroxylation and the degree of total NNK bioactivation (Figure 2.3a) or detoxification (Figure 2.3b). 7-Hydroxycoumarin formation correlated with formation of hydroxy acid (r=0.512, P<0.05) but not with other individual α-hydroxylation metabolites. Correlation analysis between the degree of NNK bioactivation and coumarin 7-hydroxylation for the high bioactivation subgroup could not be carried out because of limited tissue availability for two subjects. There was no association between NNAL formation and 7-hydroxycoumarin formation (P>0.05). However, when individuals with relatively high levels of 7-hydroxycoumarin formation (>20 fmol/mg protein/min, n=2) were excluded from analyses, a significant correlation was found (r=0.520, P<0.05) (Figure 2.3c). There was no difference in mean coumarin 7-hydroxylation activity between current and former smokers (7.25± 14.5 fmol/mg protein/min (95% CI: -0.488 to 15.00 fmol.mg protein/min) and 8.55±7.02 fmol/mg protein/min (95% CI: 3.84 to 13.3 fmol/mg protein/min), respectively). However, there was a positive relationship between age and

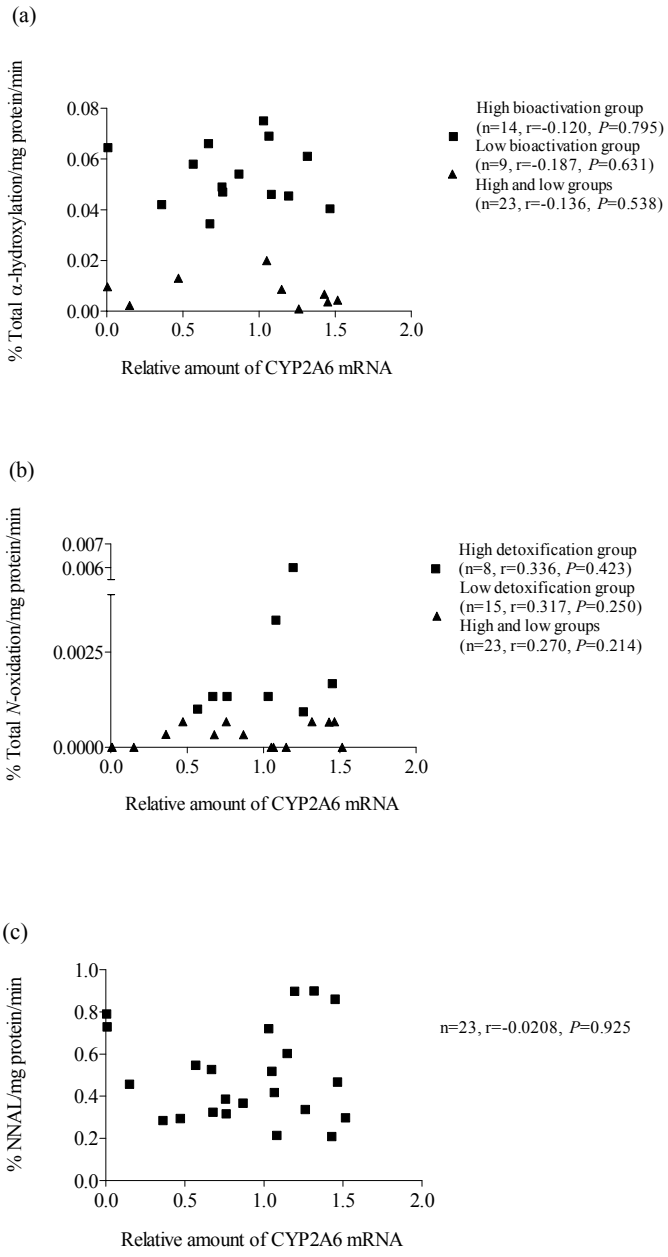

Figure 2.2 Correlation between the relative amount of CYP2A6 mRNA in human lung as determined by quantitative real-time RT-PCR and the degree of NNK: (a) total α-hydroxylation (sum of four α-hydroxylation endpoint metabolites); (b) total *N*-oxidation (sum of two pyridine-*N*-oxidation metabolites); and (c) NNAL formation for each individual (n=23, Pearson's correlation). Levels of mRNA were determined by quantitative real-time RT-PCR as described for Figure 2.1.

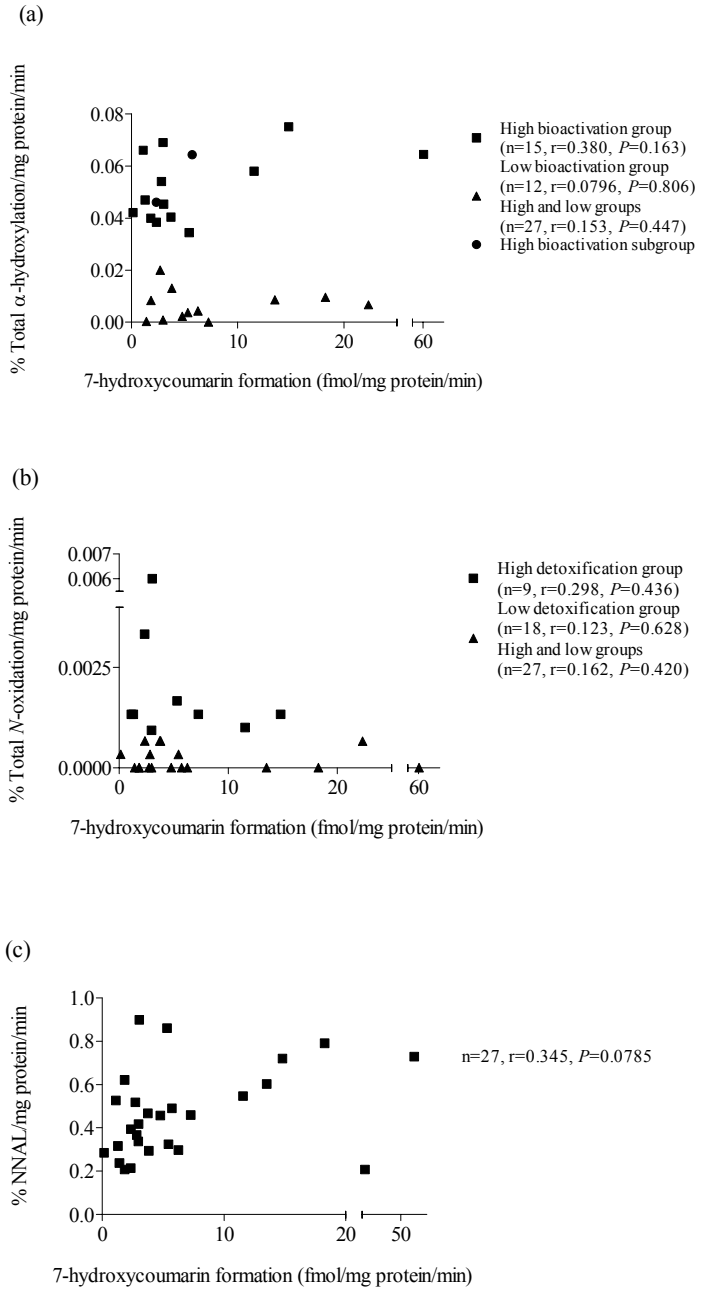

Figure 2.3 Correlation between CYP2A enzyme activity as measured by the formation of 7-hydroxycourmarin from coumarin and the degree of NNK: (a) total α-hydroxylation (sum of four α-hydroxylation endpoint metabolites); (b) total *N*-oxidation (sum of two pyridine-*N*-oxidation metabolites) for each individual; and (c) NNAL formation (n=27, Pearson's correlation).

coumarin 7-hydroxylation activity among individuals (n=27, r=0.387, $P<0.05$) and males had significantly higher activity than did females (11.6±15.0 fmol/mg protein/min, n=15 (95% CI: 3.25 to 19.9 fmol/mg protein/min) and 3.07±2.00 fmol/mg protein/min, n=12 (95% CI: 1.80 to 4.33 fmol/mg protein/min), respectively, $P<0.05$).

To determine the consistency between enzymatic activity as reflected by coumarin hydroxylation and CYP2A expression as assessed by mRNA levels, correlation analysis was performed. When results from all individuals were pooled, no significant correlations were found between 7-hydroxycoumarin formation and either CYP2A13 mRNA levels (n=26, r=-0.0685, $P>0.05$) or CYP2A6 mRNA levels (n=22, r=-0.354, $P>0.05$) (Figure 2.4).

2.3.6 Immunoinhibition of NNK Biotransformation

The presence of the CYP2A6/13 inhibitory antibody decreased total α-hydroxylation by 0.35 to 98.3 % (median decrease =10.1%) in 16 subjects' microsomes, increased it by 1.52 to 57.5 % (median increase =5.42%) in eight and had no apparent effect in microsomes from six subjects. It decreased total N-oxidation by 22.2 to 100 % (median decrease =85.0%) in 14 subjects' microsomes, increased N-oxidation by 25.3 and 200 % in two, and had no effect in 14. NNAL formation was decreased by 1.00 to 38.8 % (median decrease =8.10%) in microsomes from 19 subjects and increased by 4.33 to 50.5 % (median increase =14.6%) in ten. Regardless of whether high and low bioactivators and detoxifiers were considered separately or together, there was no significant correlation between the % change in NNK bioactivation or detoxification in

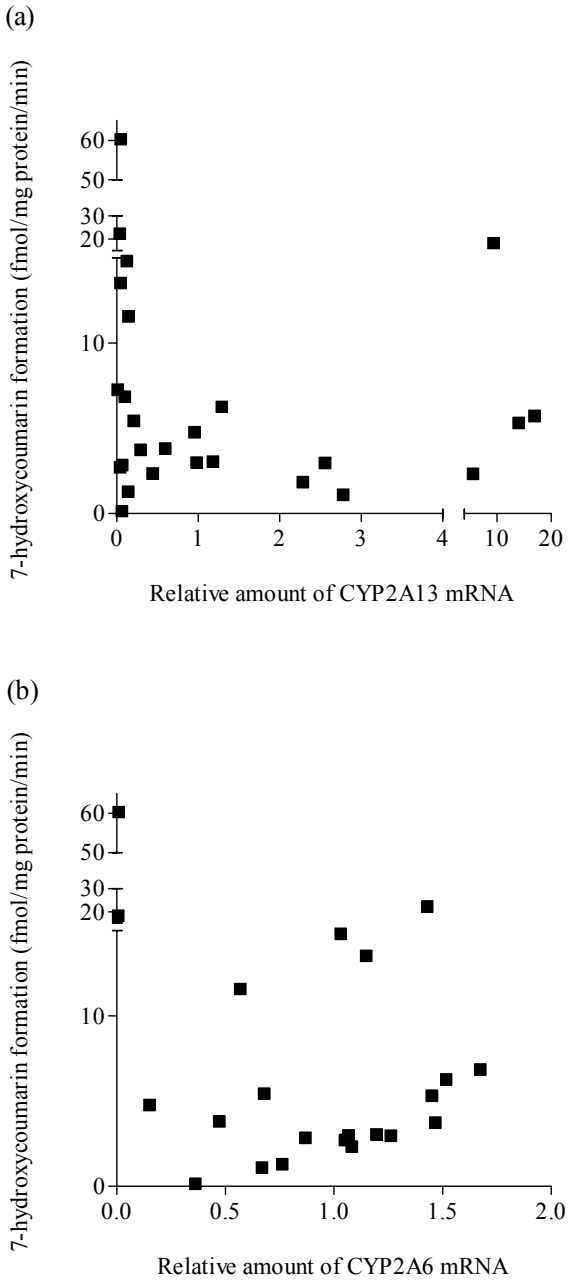

Figure 2.4 Correlation between CYP2A enzyme activity as measured by coumarin 7-hydroxylation and levels of (a) CYP2A13 mRNA and (b) CYP2A6 mRNA among individuals (n=26 and n=22, respectively, Pearson's correlation). Levels of mRNA were determined by quantitative real-time RT-PCR as described for Figure 2.1.

the presence of a CYP2A6/13 inhibitory antibody and either the degree of NNK metabolism formation of individual α-hydroxylation or N-oxidation metabolites (Table 2.3). There also was no significant correlation between NNAL formation and the % change in NNAL formation in the presence of a CYP2A6/13 inhibitory antibody (Table 2.3).

When assessing the consistency between CYP2A immunoinhibition-mediated changes in NNK metabolism and the measures of assessing CYP2A expression or activity, no significant correlations were found between levels of CYP2A13 or CYP2A6 mRNA and CYP2A6/13 immunoinhibition-mediated changes in total NNK bioactivation, total NNK detoxification, or NNAL formation. Similarly, no significant correlations were found between 7-hydroxycoumarin formation and CYP2A6/13 immunoinhibition-mediated changes in total NNK bioactivation, total NNK detoxification, or NNAL formation (Table 2.3).

2.3.7 Effects of *CYP2A13* Arg257Cys Polymorphism on NNK Biotransformation

Following PCR-RFLP analysis, homozygous wildtype (C/C) samples produced the anticipated products of 217 bp and 158 bp (Wang *et al.*, 2003a), whereas heterozygous variant (C/T) samples produced products of 375 bp, 217 bp and 158 bp. The resultant genotype frequencies were 83/84 (98.8 %) C/C, 1/84 (1.2 %) C/T and 0/84 (0%) T/T. NNK metabolite profile for the one heterozygous variant (0.047 % total α-hydroxylation/mg protein/min; 0.00133 % total *N*-oxidation/mg protein/min; 0.316 % NNAL formation/mg protein/min) did not differ from those of the C/C subjects.

Table 2.3 Correlation analysis between the extent of CYP2A immunoinhibition and the degree of NNK biotransformation, levels of CYP2A mRNA and 7-hydroxycoumarin formation

		% Change in total α-hydroxylation[a]		% Change in total *N*-oxidation		% Change in NNAL formation	
Total α-hydroxylation (n=29)		r=0.234[b]	*P*=0.222[b]				
	High bioactivation group	r=0.285	*P*=0.268				
	Low bioactivation group	r=0.313	*P*=0.322				
Total *N*-oxidation (n=29)				r=0.177	*P*=0.360		
	High detoxification group			r=0.218	*P*=0.573		
	Low detoxification group			r=0.156	*P*=0.512		
NNAL formation (n=29)						r=-0.0362	*P*=0.852
CYP2A13 mRNA (n=28)		r=0.0861	*P*=0.633	r=0.0586	*P*=0.767	r=0.112	*P*=0.569
CYP2A6 mRNA (n=24)		r=-0.338	*P*=0.107	r=0.0815	*P*=0.705	r=-0.0234	*P*=0.960
7-Hydroxycoumarin formation (n=27)		r=0.0200	*P*=0.920	r=0.125	*P*=0.533	r=0.00856	*P*=0.966

[a] CYP2A6/13 immunoinhibition was determined by the % change in NNK metabolism in the presence of a CYP2A6/13 inhibitory antibody. Immunoinhibition experiments were performed independently of other experiments that assessed NNK metabolism, mRNA expression and coumarin 7-hydroxylation.

[b] Correlation coefficients (r) and *P* values determined using Pearson's correlation analysis.

2.4 DISCUSSSION

Although we have previously used inhibitors to assess CYP2A6 contributions to NNK metabolism in adult human lung (Smith *et al.*, 2003), this is the first study to assess CYP2A13 contributions. Examination of CYP2A13-mediated NNK metabolism was of interest as this human CYP is expressed predominantly in the lung (Su *et al.*, 2000) and is the most catalytically active CYP isoform in the metabolic activation of NNK (Su *et al.*, 2000). Our grouped results suggest no correlation between the degree of NNK bioactivation or detoxification and CYP2A gene expression and activity. However, a subgroup of individuals was identified for whom high levels of NNK bioactivation correlated with high CYP2A13 mRNA levels (Figure 2.1), suggesting that CYP2A13 may contribute substantially to NNK bioactivation in some, but not all individuals. Similarly, for NNK detoxification, a subgroup of individuals was identified for whom CYP2A13 mRNA expression correlated with detectable levels of NNAL-*N*-oxide. However, the pooled results suggest that CYP2A13 is not the sole enzyme contributing to NNK metabolism in peripheral human lung microsomes. The apparent discrepancy between our results and those reported for fetal nasal microsomes (Wong *et al.*, 2005) may reflect the fact that levels of CYP2A13 mRNA (Su *et al.*, 2000) and CYP2A13 immuno-reactive protein (Zhu *et al.*, 2006) are considerably higher in the nasal mucosa than in the lung. Hence, in areas of the respiratory system where CYP2A13 levels are lower, other enzymes may contribute substantially to NNK metabolism.

Based on the interindividual variability in NNK metabolism, individuals were classified into high or low bioactivation and detoxification groups. The observation of

distinct groups is consistent with our previous study of seven subjects (Smith *et al.*, 2003). The distribution of subjects between bioactivation categories is not consistent with reported frequencies of established genetic polymorphisms of enzymes that have been characterized with respect to NNK metabolism, suggesting the possibility that environmental factors, rather than genetics, make the largest contribution to interindividual variability in NNK metabolism.

Although the levels of total NNK-derived metabolites were markedly different between the high and low bioactivation groups, the levels of total NNAL-derived metabolites were not significantly different (Table 2.2). This was unexpected because it was anticipated that the NNAL-derived metabolites would vary between the groups to the same extent as the NNK-derived metabolites, since both the high and low bioactivation groups formed equal amounts of NNAL. The differences in NNK and NNAL metabolism may be due to differences in the affinity of different CYP isoforms for NNK versus NNAL as a result of differences in polarity between the keto group of NNK and the hydroxyl group of NNAL. In fact, Jalas *et al.* (2003) found that CYP2A enzymes are more efficient catalysts of NNK metabolism than of NNAL metabolism.

CYP involvement in human pulmonary microsomal NNK metabolism was supported by NADPH-dependence but as consistent with previous studies (Smith *et al.*, 1992a; Smith *et al.*, 1995) sensitivity to CO inhibition was variable. The fact that removal of the NAPDH-generating system and CO treatment did not completely inhibit NNK metabolism in some individuals suggests that non CYP enzymes may also be involved in the metabolism.

Consistent with previous results (Smith *et al.*, 2003; Maser *et al.*, 2000), NNAL formation from NNK was NADPH-dependent for all subjects, but our results are the first to suggest a role for CYP2A13 in catalyzing the carbonyl reduction of NNK to form NNAL. This role is supported by our finding that NNAL formation positively correlated with CYP2A13 mRNA levels, although this correlation was dependent upon inclusion of individuals with high levels of CYP2A13 mRNA. It is also supported by the observation that NNAL formation positively correlated with the degree of 7-hydroxycoumarin formation in all individuals with the exception of those with relatively high coumarin 7-hydroxylation activity. It is possible that coumarin 7-hydroxylation is mainly CYP2A6-mediated in individuals with relatively high levels 7-hydroxycoumarin formation. It has been suggested that the microsomal enzyme, 11β-hydroxysteroid dehydrogenase (11β-HSD) rather than P450s, is the major catalyst of this reaction (Hecht, 1998). However, in microsomes from human lung, NNAL formation was insensitive to an 11β-HSB inhibitor (Breyer-Pfaff *et al.*, 2004) and sensitive to P450 inhibitors. In contrast to results from the present study, an anti-CYP2A antibody did not inhibit NNAL formation in human fetal nasal microsomes (Wong *et al.*, 2005). However, since fetal tissues had been pooled, interindividual variability in CYP2A13 contributions to NNAL formation could not be assessed. As well, age is known to affect P450 activity (Day *et al.*, 2006).

Results presented here are also the first to support involvement of CYP2A13 in NNAL detoxification in human lung tissue for some individuals. While no significant correlations were found between CYP2A13 mRNA and either total NNK detoxification or NNK-*N*-oxide levels, an association was found between levels of NNAL-*N*-oxide and

CYP2A13 mRNA. This result is consistent with the finding that heterologously-expressed CYP2A13 is capable of catalyzing NNAL *N*-oxidation but not NNK *N*-oxidation (Jalas *et al.*, 2003).

No correlations were found between the degree of total NNK bioactivation or detoxification and 7-hydroxycoumarin formation, the latter of which is expected to reflect overall CYP2A activity. However, assessing associations between CYP2A13 or CYP2A6 activity and NNK metabolism is difficult since available substrates with the exception of the drug phenacetin, do not discriminate between CYP2A13 and CYP2A6, and because CYP2A6 is 10 times more active than CYP2A13 at catalyzing coumarin 7-hydroxylation (Su *et al.*, 2000). While recently it was shown that CYP2A13 can efficiently metabolize phenacetin and CYP2A6 has virtually no catalytic activity towards this drug (Fukami *et al.*, 2007), phenacetin is also a CYP1A2 substrate. Since CYP1A2 can also potentially bioactivate NNK (Smith *et al.*, 1996) and is expressed in human lung (Wei *et al.*, 2001), using phenacetin to assess CYP2A13 activity in human lung microsomes would also be problematic. When examining associations between individual α-hydroxylation metabolites and 7-hydroxycoumarin formation, a significant correlation was found between the degree of hydroxy acid formation and 7-hydroxycoumarin. This finding is consistent with both CYP2A13 and CYP2A6 being more efficient catalysts of α-methylene hydroxylation compared to α-methyl hydroxylation (Su *et al.*, 2000; Jalas *et al.*, 2003). Since neither CYP2A13 nor CYP2A6 mRNA correlated with hydroxy acid formation, the relative contribution of these isoforms is not known.

The absence of significant correlations between the degree of NNK bioactivation, detoxification or keto reduction and CYP2A immunoinhibition may also reflect the fact that specific contributions of either CYP2A13 or CYP2A6 to NNK metabolism are difficult to assess since commercially available inhibitory antibodies do not discriminate between CYP2A13 and CYP2A6 (Su *et al.*, 2000). Moreover, an attempt was made to determine the relative CYP2A protein levels among individuals by immunoblotting; however protein levels were too low for detection. While our results do not suggest uniform involvement of CYP2A13 in NNK metabolism across all individuals, the possibility exists that more specific measures of enzyme contributions would reveal that a correlation exists for a greater proportion of individuals.

In some microsomal samples, the presence of the CYP2A6/13 inhibitory antibody increased levels of NNK metabolism. It has been suggested that non-uniform constitution of human microsomes between incubates and cigarette smoke particulate matter might affect how NNK biotransformation activities are expressed in different incubates (Smith *et al.*, 2003).

No statistically significant correlations were found between CYP2A13 or CYP2A6 mRNA levels and 7-hydroxycoumarin formation when tissues from all patients were considered (Figure 2.4). The lack of associations may be due to the fact that 7-hydroxycoumarin formation is a measure of overall CYP2A enzyme activity, not specifically CYP2A13 or CYP2A6 (Su *et al.*, 2000). In addition, nicotine is a mechanism-based inactivator of both CYP2A13 and CYP2A6 (von Weymarn *et al.*, 2006), so current smokers could potentially have had diminished CYP2A enzyme

activity. However, a higher proportion of current smokers were actually high bioactivators as compared to low bioactivators and levels of 7-hydroxycoumarin formation did not differ between current and former smokers. There also were no significant correlations between the CYP2A immunoinhibition of NNK metabolism and CYP2A13 mRNA levels or CYP2A6 mRNA levels or 7-hydroxycoumarin formation (Table 2.3). Inconsistency between these parameters could be attributed to a lack of specificity of the antibody for either CYP2A isoform (e.g. according to information from the supplier, the antibody reacts with CYP2A6, CYP2A13 and mildly with CYP2E1).

A limitation of the present study is that a correlational approach was used to assess CYP involvement. While this approach provides important information about the degree of association between different parameters, it does not necessarily establish a cause and effect relationship.

Genotype frequencies for the *CYP2A13* Arg257Cys polymorphism were consistent with those previously published for a Caucasian population (96.2% (C/C), 3.8% (C/T) and 0% (T/T) (Zhang *et al.*, 2002). While NNK metabolism for the one heterozygous variant did not differ from other subjects, it is not possible to accurately determine the actual contribution of this polymorphism to NNK metabolism, since only one variant was found. The current study did not assess the influence of *CYP2A6* polymorphisms on NNK metabolism since no clear associations have been found between *CYP2A6* polymorphisms and lung cancer risk (Raunio *et al.*, 2001).

In summary, examination of the interindividual differences in the pulmonary microsomal metabolism of NNK revealed the existence of high and low bioactivation and detoxification groups. Differences in bioactivation and detoxification between groups correlated with differences in the oxidation of NNK but not of NNAL. If the basis for these differences could be determined, identification of high and low categories for bioactivation and detoxification may be useful for predicting susceptibility of individuals to NNK-induced carcinogenesis. Results from this study are the first to suggest a role for CYP2A13 in NNAL formation. Although results do not support a role for either CYP2A13 or CYP2A6 as a major contributor to NNK bioactivation or detoxification in all individuals, results from subgroups of individuals suggest that CYP2A13 contributes to NNK metabolism, but this contribution varies between individuals.

CHAPTER 3

REPAIR OF 4-(METHYLNITROSAMINO)-1-(3-PYRIDYL)-1-BUTANONE-INDUCED DNA PRIDYLOXOBUTYLATION BY NUCLEOTIDE EXCISION REPAIR

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3.1 INTRODUCTION

To exert carcinogenicity, NNK is metabolically activated by α-carbon hydroxylation to form DNA-reactive species via two different pathways (Figure 1.2). α-Methyl carbon hydroxylation generates 4-(hydroxymethylnitrosamino)-1-(3-pyridyl)-1-butanone, which decomposes to 4-oxo-4-(3-pyridyl)-1-butanediazohydroxide. This compound pyridyloxobutylates DNA (Figure 1.5). The compound, 4-(acetoxymethylnitrosamino)-1-(3-pyridyl)-1-butanone (NNKOAc), a chemically activated form of NNK, is used experimentally because it forms POB adducts exclusively, analogous to those formed upon reaction of NNK with DNA. Under hydrolytic conditions, unstable POB adducts will decompose to yield 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB) (Hecht *et al.*, 1988) (Figure 1.5). α-Methylene carbon hydroxylation leads to formation of 4-hydroxy-4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, which decomposes to methanediazohydroxide, which in turn methylates DNA (Figure 1.5).

NNK-induced DNA methylation occurs at the 7- and O^6-positions of guanine and at the O^4-position of thymidine (Hecht *et al.*, 1986; Belinsky *et al.*, 1986), while NNK-

induced pyridyloxobutylation occurs at the N^7-, N^2- and O^6-positions of guanine and O^2 positions of cytosine and thymine (Wang *et al.*, 2003b; Sturla *et al.*, 2005; Lao *et al.*, 2006).

It is believed that the primary repair mechanism by which O^6-POB-dGuo and O^6-mG adducts are removed from DNA is via O^6-alkylguanine-DNA alkyltransferases (AGTs) (Wang *et al.*, 1997). However, other than AGTs, repair pathways involved in the removal of POB adducts have not been identified. We hypothesized that since POB adducts are bulky, they would distort the DNA helix sufficiently to be recognized by nucleotide excision repair (NER).

In the present study, we determined if NER is involved in POB adduct repair by assessing the ability of cell-free nuclear protein extracts from NER-deficient XP cell lines to repair NNK-induced POB adducts and by determining the relative susceptibilities of normal and NER-deficient cell lines to NNKOAc cytotoxicity.

3.2 MATERIALS AND METHODS

3.2.1 Cell Lines

Human lymphoid cell lines were obtained from Coriell Cell Repositories (Camden, NJ). The XPA (GM02250) and XPC (GM02246) cell lines were derived from xeroderma pigmentosum (XP) patients belonging to complementation groups A and C respectively, and the normal cell line (GM01953) was derived from an apparently healthy individual. Cultures were grown in RPMI 1640 medium supplemented with 15% heat inactivated fetal bovine serum.

3.2.2 Preparation of Cell-Free Nuclear Protein Extracts

Cell-free protein extracts from XPA, XPC and normal cell lines were prepared by the method of Wood *et al.* (1988) with slight modifications. (1) One half a liter of cells at 5-9 x 10^5 cells/mL was used for each preparation. (2) Protease inhibitors were added (final concentrations, 0.14 mg/mL phenyl methyl sulfonyl fluoride, 2.0 μg/mL leupeptin and 20 μg/mL aprotinin). Protein content of extracts was determined by the method of Lowry *et al.* (1951). Extracts typically contained 6-9 mg/mL protein. Extracts were snap-frozen in liquid nitrogen and stored at –80°C.

3.2.3 Preparation and Analysis of Pyridyloxobutylated Plasmid DNA

A 2961 bp plasmid derived from pBluescript SK+ (Stratagene, La Jolla, CA) was grown in E. coli DH5α in LB broth and isolated using a Qiagen Plasmid Mega Kit (Qiagen, Valencia, CA). The method to prepare pyridyloxobutylated DNA was adapted from Peterson *et al.* (1993) and Wang *et al.* (2003b). NNKOAc (20 mM, Toronto Research Chemicals, North York, ON) was reacted with 1.04 mg/mL pBluescript SK+ plasmid DNA in a 250 μL reaction mixture containing sodium citrate buffer (final concentration, 15 mM sodium citrate, pH 7, 1 mM EDTA) and 4 U/mL porcine liver esterase (Sigma-Aldrich Co., St. Louis, MO). After 1 h at room temperature, NaCl (final concentration, 0.33 M) was added and DNA was precipitated with isopropanol, washed with 70 % ethanol, dried and redissolved in TE buffer (10 mM Tris HCl, pH 8, 1 mM EDTA).

To determine the degree of plasmid adduction, pyridyloxobutylated plasmid DNA was subjected to strong acid hydrolysis (0.8 N HCl, 80 °C, 3 h) (Wang *et al.*, 2003b) and

levels of released HPB were analyzed by gradient reverse phase HPLC with UV detection (Smith *et al.*, 2003). HPB levels were quantified by standard curve interpolation using standard HPB (Toronto Research Chemicals, North York, ON).

The number of apurinic/apyrimidinic (AP) sites generated as a result of pyridyloxobutylation of plasmid DNA was measured using a DNA Damage Quantification Kit for AP site counting (Dojindo Molecular Technologies Inc., Gaithersburg, MD) according to manufacturer's protocol. Briefly, undamaged and pyridyloxobutylated plasmid DNA were treated with Aldehyde Reactive Probe (ARP) reagent which tagged AP sites with biotin residues. ARP-biotin sites were quantified by colorimetric detection of horseradish peroxidase conjugated to avidin. The number of AP sites on each plasmid was determined by interpolation of a standard curve produced using DNA with known numbers of ARP-DNA sites per 1 x 10^5 bp.

3.2.4 Characterization of Plasmid Pyridyloxobutyl Adducts

Analyses of pyridyloxobutylated and untreated plasmid DNA were carried out by reverse phase HPLC-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) as described previously (Sturla *et al.*, 2005; Lao *et al.*, 2006), by Dr. Peter Villalta at the University of Minnesota (Minneapolis, MN). Specifically, we assessed the relative levels of the POB adducts, 7-POB-Gua, O^2-POB-Cyt, O^2-POB-dThd and O^6-POB-dGuo. The recovery of all four adducts during sample preparation is similar (Sturla *et al.*, 2005). To prepare plasmid DNA samples for LC-ESI-MS/MS, samples were enzymatically digested as described (Lao *et al.*, 2006). Briefly, samples containing

approximately 0.26 mg plasmid DNA were redissolved in 10 mM sodium succinate/5 mM $CaCl_2$ buffer (pH 7.0) and digested with 75 U micrococcal nuclease (Worthington Biochemical Corp., Lakewood, NJ), 450 mU phosphodiesterase II (Worthington Biochemical Corp., Lakewood, NJ) and 150 U alkaline phosphatase (Roche Molecular Biochemicals, Indianapolis, IN). Hydrolysates were purified by centrifugation using an Ultrafree-MC 30 kDa centrifugal filter device (Millipore Corp., Bedford, MA) followed by SPE as described (Lao *et al.*, 2006) except purification was carried out using a Sep-Pak Plus C18 cartridge (Waters Corp., Milford, MA).

3.2.5 Preparation of UV-Damaged Plasmid DNA

pBluescript SK+ plasmid DNA at 0.1 mg/mL in TE buffer was irradiated with 254 nm UV light at a fluence of 500 J/m^2, producing approximately 13 cyclobutane pyrimidine dimers per plasmid (Wood *et al.*, 1988).

3.2.6 *In vitro* DNA Repair Synthesis Assay

The repair synthesis assay was performed as described (Wood *et al.*, 1995; Wood *et al.*, 1988) with slight modifications (Figure 3.1). The 50 µL reaction mixture contained 400 ng of pyridyloxobutylated plasmid DNA, UV-damaged plasmid DNA or undamaged plasmid DNA, 40 mM HEPES-KOH (pH 7.8), 0.8 mM DTT, 4.0 µmol/L dTTP, 20 µM of each dGTP, dCTP and dATP, 23 mM phosphocreatine, 18 µg bovine serum albumin (nuclease-free, Sigma), 2.5 µg creatine phosphokinase, 2.0 mM ATP, 5.0 mM $MgCl_2$, 0.4 mM EDTA, 70 mM KCl, 80 µg protein extract and 2.0 µCi [α^{32}P]dTTP

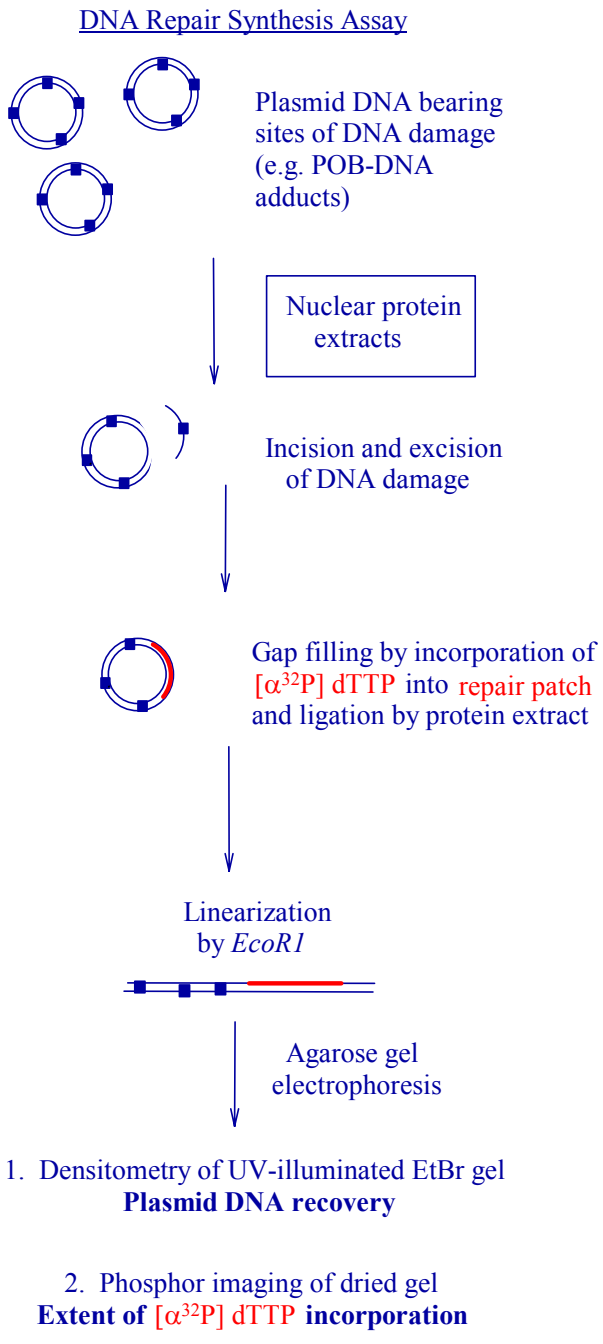

Figure 3.1 Scheme of the *in vitro* DNA repair synthesis assay on damaged plasmid DNA by nuclear protein extracts isolated from cells and whole tissue. Squares on plasmid represent DNA lesions (modified from Salles *et al*., 1995a). POB: pyridyloxobutyl; dNTPs: triphosphates of nucleosides; EtBr: ethidium bromide.

(Amersham). All samples, excluding those of the time course experiments, were incubated for 3 hours at 30°C. Reactions were terminated by adding EDTA (final concentration, 20 mM). Following incubation with 7.0 mg/mL RNase (37°C for 10 minutes) and 925 μg/mL proteinase K with 0.5% SDS (65°C for 30 minutes), plasmid DNA was purified by extraction with phenol/chloroform/isoamyl alcohol (25:24:1; v/v/v), precipitated with 100% ice-cold ethanol, washed in 80% ethanol, dried and linearized with 1 U of EcoR1 (New England Biolabs, Beverly, MA). Plasmids were electrophoresed on a 1% agarose gel. The repair synthesis assay was performed in duplicate for each protein extract and results are presented as means of duplicate samples.

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 [α^{32}P]dTTP incorporation was determined by phosphor imaging (Molecular Dynamics, Piscataway, NJ) of the dried gel (Figure 3.2).

3.2.7 Assessment of NNKOAc-Induced Cytotoxicity

Approximately 6 x 10^4 cells (normal, XPA or XPC) for each treatment were centrifuged at 3000 x g for 4 min and the pellet resuspended in 125 μL of culture medium (0.5 x 10^6 cells/mL). Normal, XPA and XPC cells were then treated with either 25 μM NNKOAc dissolved in dimethylsulfoxide (DMSO) (final concentration 0.5%) or DMSO alone, and incubated for 4 h at 37 ºC in a cell incubator. Cytotoxicity was assessed by trypan blue exclusion using 0.5% trypan blue.

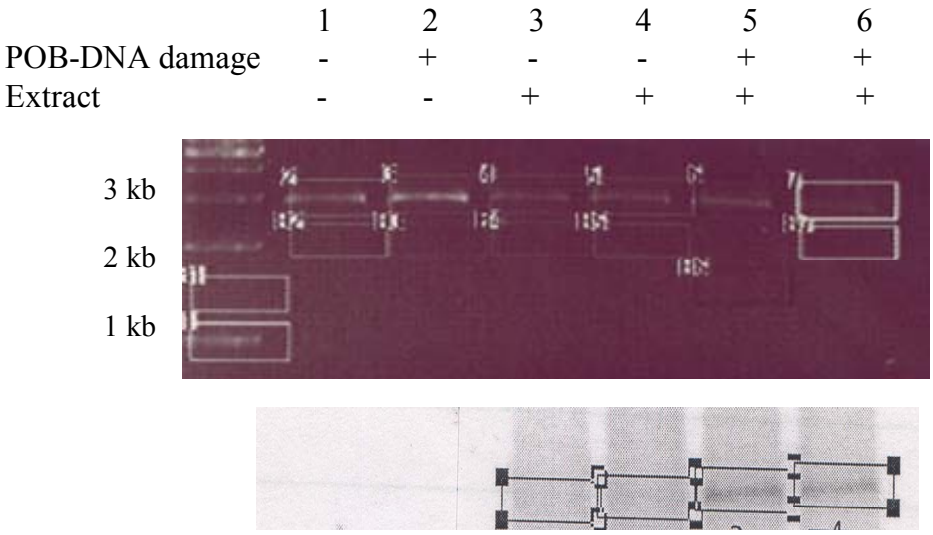

Figure 3.2 UV-illuminated ethidium bromide stained agarose gel of linearized plasmid DNA and phosphor image demonstrating the ability of cell-free nuclear protein extracts to catalyze *in vitro* DNA repair synthesis. Repair synthesis (evidenced by the incorporation of [α^{32}P] dTTP into plasmid DNA) is not detectable in the absence of extract (lanes 1 and 2). Lanes 3 and 4 demonstrate non-specific background repair synthesis activity towards undamaged plasmid DNA. Lanes 5 and 6 show pyridyloxobutylation (14 adducts/plasmid) damage-specific repair synthesis.

3.3 RESULTS

3.3.1 Characterization of Pyridyloxobutylated Plasmid DNA Substrate for Repair Analysis

Using HPB release to estimate the extent of plasmid adduction, substrates were generated with approximately 14 adducts/plasmid. However, this value is an estimate, since not all POB adducts release HPB upon neutral thermal hydrolysis (Sturla *et al.*, 2005) and HPB can also be released from DNA phosphate adducts (Haglund *et al.*, 2002). Also, POB adduct number was based on adduction of guanine, since the major POB adduct formed *in vitro* and *in vivo* is 7-POBGua (Wang *et al.*, 2003b; Sturla *et al.*, 2005; Lao *et al.*, 2006). However, POB adducts have also been identified on cytosine and thymine (Wang *et al.*, 2003b; Sturla *et al.*, 2005).

The adducted plasmid was analyzed for the presence and relative levels of the four major POB adducts. The adducts 7-POB-Gua, O^2-POB-Cyt, O^2-POB-dThd and O^6-POB-dGuo comprised 31.2, 22.7, 25.5 and 20.6 %, respectively of the total POB adducts monitored (Figure 3.3).

Since POB adducts 7-POB-dGuo and O^2-POB-dCyd have potential to form AP sites (Lacoste *et al.*, 2006), the number of AP sites on each plasmid were quantified. Undamaged and pyridyloxobutylated plasmid DNA contained 0.07 and 0.8 AP sites/plasmid, respectively.

Gel electrophoresis of the DNA substrate revealed that treatment of plasmid DNA

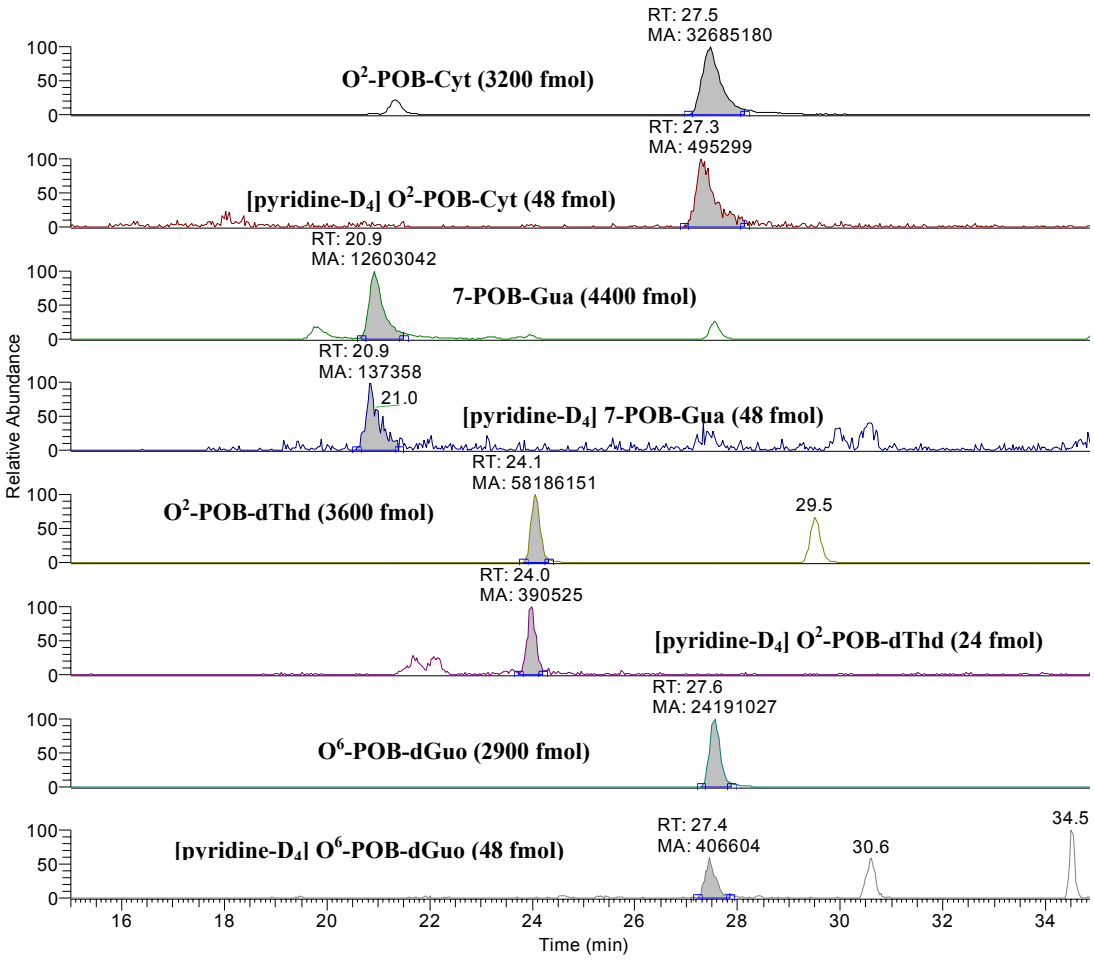

Figure 3.3 Selected reaction monitoring chromatograms obtained upon LC-ESI-MS/MS analysis of individuals POB-DNA adducts from enzymatic hydrolysates of pyridyloxobutylated plasmid DNA that had reacted with NNKOAc and of individual deuterated standards. RT: retention (minutes); MA: MS peak area. Levels of individual POB-DNA adducts were calculated by dividing the MA of each adduct by the MA produced with a known amount of its deuterated standard.

with NNKOAc produced single-strand nicks, leaving approximately 90% of the plasmid in the supercoiled form. Pyridyloxobutylated plasmid DNA substrates were found to be stable under the experimental conditions used for the repair synthesis assay as demonstrated by absence of release of HPB (lower limit of detection for HPB was < 5 $x10^{-11}$ mol and average HPB release from POB adducted DNA following acid hydrolysis was 200 times greater) from incubates conducted in the absence of nuclear protein extracts.

3.3.2 Repair of Pyridyloxobutylated Plasmid DNA by NER-Deficient Nuclear Protein Extracts

Time course analysis of repair synthesis activity of NNK-induced pyridyloxobutylation and UV-induced DNA damage by normal and deficient cell lines revealed that repair activity appeared to plateau between 3 and 6 hours (Figure 3.4), so subsequent incubations were carried out for 3 hours. At 3 hours, repair activity of UV damaged DNA by nuclear extracts from XPA and XPC cell lines was 42% and 66% lower, respectively, than repair activity by normal cell line extract (Figure 3.4a). Similarly, XPA and XPC extracts had 61 % and 70 % lower repair activities of pyridyloxobutylated DNA than did normal cell extract (Figure 3.4b).

Normal cell extract was ~3 times more active at repairing POB adducts compared to UV-induced adducts at 3 h (4140 ± 225 amol [α^{32}P]dTTP incorporated/mg of DNA and 953 ± 163 amol [α^{32}P]dTTP incorporated/mg of DNA, respectively) (Figure 3.4).

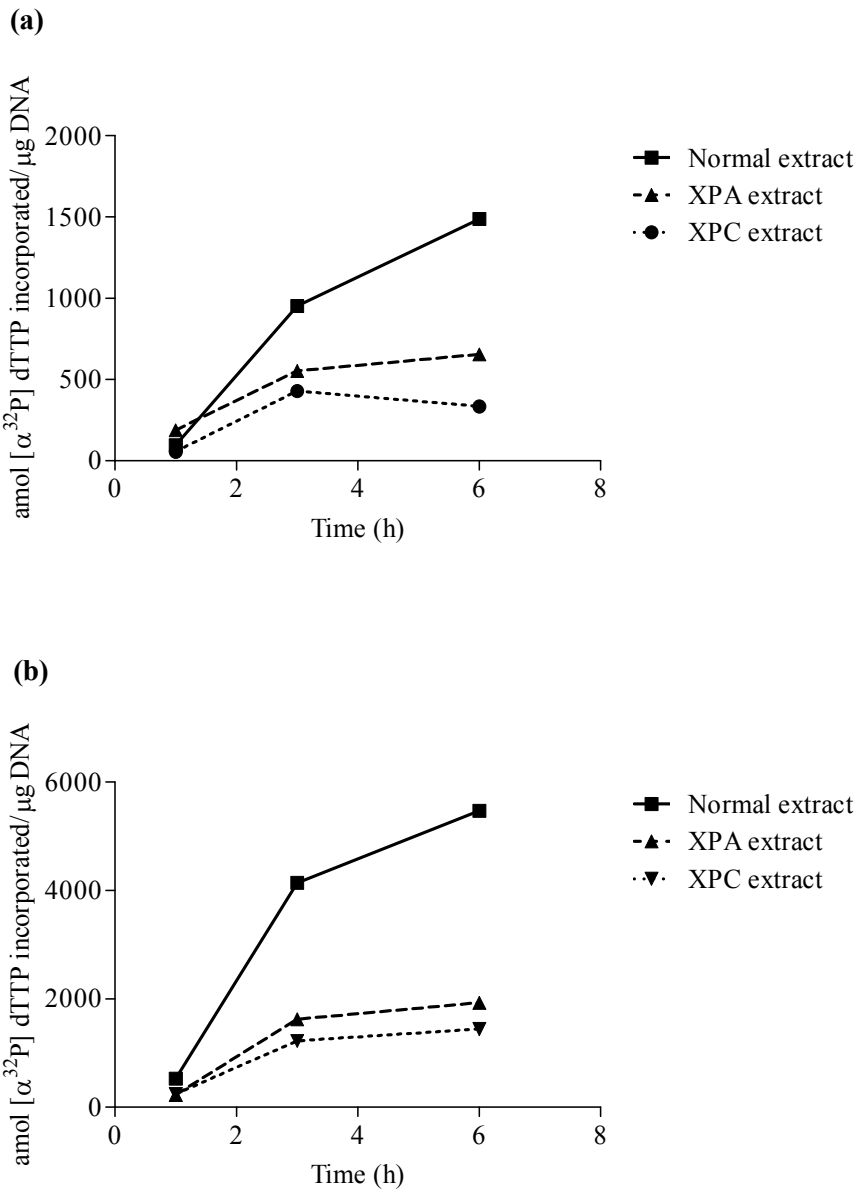

Figure 3.4 *In vitro* DNA repair synthesis of (a) UV-induced DNA damage (~13 adducts/plasmid) and (b) NNK-induced pyridyloxobutyl (POB) DNA damage (~14 adducts/plasmid) by protein extracts (80 µg) isolated from normal and NER-deficient, XPA and XPC cell lines after 1, 3 and 6 h incubation. Radioisotope incorporation into undamaged DNA has been subtracted from damage-specific incorporation**.**

Repair activity of normal cell extracts was nondetectable (<0.3 amol [α^{32}P]dTTP incorporated/mg of DNA) in the absence of ATP.

3.3.3 *In vitro* Complementation of POB Adduct Repair Activity by Combining NER-Deficient Protein Extracts

When XPA and XPC protein extracts (40 μg each) were combined, UV damage repair activity increased to approximately the same level of repair achieved when 40 μg of normal cell protein extract was used (figure 3.5a). Furthermore, repair activities of the combined XPA and XPC extracts (40 μg each) and 40 μg of normal cell extract were approximately 50% lower than the level of repair than that of 80 μg of normal cell extract. Similarly, repair activity of pyridyloxobutylated DNA was increased when XPA and XPC extracts were combined (40 μg) relative to repair activities of XPA or XPC extracts (80 μg) alone (Figure 3.5b). Although, repair activity was higher in the combined extracts versus 80 μg of either XPA or XPC extract in experiments using both UV-damaged and pyridyloxobutylated substrates, reconstituted repair activity was greater for the pyridyloxobutylated DNA.

3.3.4 NNKOAc-Induced Cytotoxicity

Viability in DMSO-treated cells was 75, 88 and 83% for XPA, XPC and normal cells, respectively, and was not affected by DMSO. Treatment with 25 μM NNKOAc decreased cell viability in all three cell types but to a greater degree in XPA and XPC cells than in normal cells (by 30% and 45 % and 20% respectively) (Figure 3.6). Since

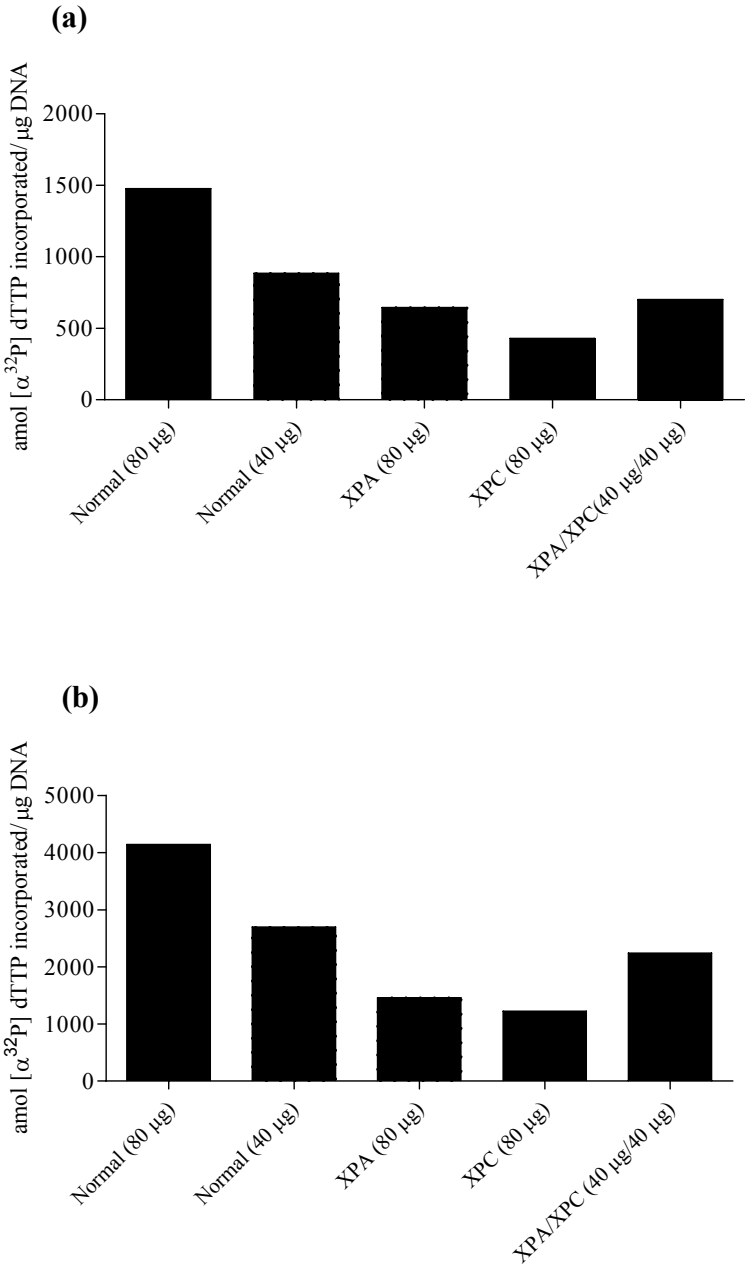

Figure 3.5 The effect of combining NER-deficient protein extracts from XPA and XPC complementation groups (total 80 µg protein per reaction) on the *in vitro* DNA repair synthesis activity of (a) UV-induced DNA damage (~ 13 adducts/plasmid) and (b) NNK-induced pyridyloxobutylated DNA (~14 adducts/plasmid) at 3h. Radioisotope incorporation into undamaged DNA has been subtracted from damage-specific incorporation.

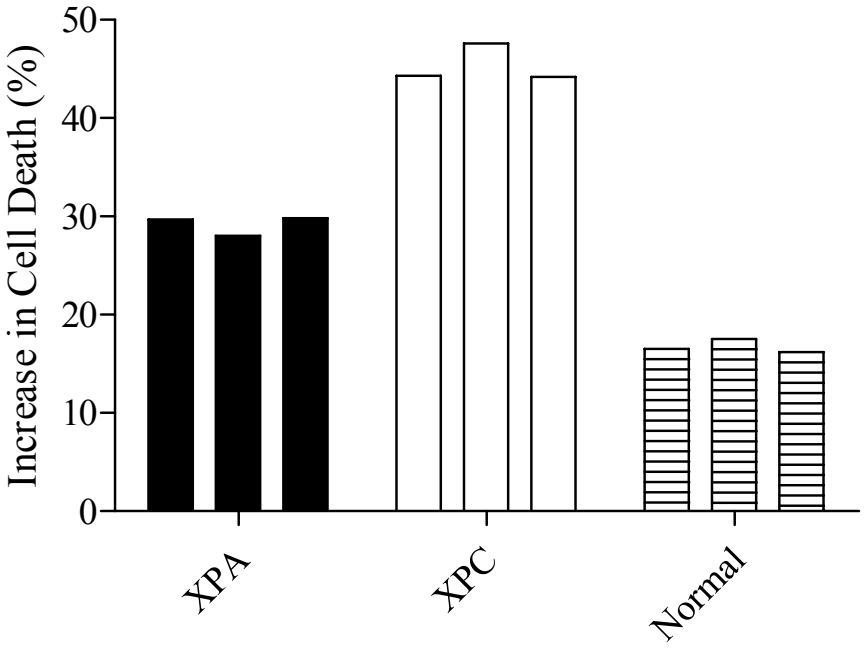

Figure 3.6 Changes in cell viability in normal and NER-deficient XPA and XPC lymphocytes after 4 h of treatment with 25μM NNKOAc. Cell viability was assessed by trypan blue exclusion and changes in viability were calculated by subtracting cell viability with NNKOAc treatment by cell viability with DMSO (control) treatment. Triplicate values are shown for each cell type and are representative of values obtained from two independent experiments.

only two independent experiments (n=2) assessing cytotoxicity were carried out, statistical analysis of the data was not performed.

3.4 DISCUSSION

This is the first study to demonstrate involvement of NER in the repair of POB adducts. A role for NER in the repair of POB adducts is supported by our findings thatboth XPA deficient and XPC deficient extracts showed reduced repair activity towards pyridyloxobutylated DNA and that as expected, combining of XPA and XPC extracts enhanced repair activity (Wood *et al.*, 1988). The XP proteins play a key role in the damage recognition and incision steps of NER (Wood, 1997), with XPA and XPC being critical in the recognition step. Moreover, unlike other NER proteins that can be involved in other DNA repair processes, XPA and XPC are only involved in NER, with XPC being involved in only GGR and XPA being involved in both GGR and TCR. These results were verified with UV damaged DNA, which is known to be repaired by NER (Wood *et al.*, 1988). It has been found that NER on plasmid DNA resembles genomic repair *in vivo* (Salles *et al.*, 1995a) and defective repair has been observed in extracts from repair deficient XP cells (Wood *et al.*, 1988; Aboussekhra *et al.*, 1995).

Involvement of NER in POB adduct repair was also supported by the fact that XPA and XPC cells were about 2.3 and 1.5 times more susceptible to the cytotoxicity of NNKOAc. Although one cannot preclude the possibility that other mechanisms are involved, the enhanced susceptibility of XP cells to NNKOAc cytotoxicity is consistent with their decreased ability to repair NNKOAc-induced DNA pyridyloxobutylation.

The involvement of NER in POB adduct repair may provide insight into an earlier observation made by Thomson *et al.* (2003). A/J mouse AGT repairs O^6-POB-dGuo and O^6-mG with equal efficiency (Thomson *et al.*, 2003); however lower levels of O^6-POB-dGuo than of O^6-mG have been found in A/J mouse lung following *in vivo* treatment with NNK (Thomson *et al.*, 2003). Since NER is involved in the repair of POB adducts and potentially involved in the repair of O^6-POB-dGuo, lower levels of O^6-POB-dGuo *in vivo* may be due to efficient adduct removal of O^6-POB-dGuo by both AGTs and NER.

Although an earlier study (Cloutier and Castonguay, 1998) demonstrated the involvement of base and/or excision repair pathways in the repair of NNK-induced DNA damage, direct involvement of NER could not be established since findings were based on the use of DNA polymerase inhibitors which affect not only NER but also other repair pathways including base excision repair (BER) and mismatch repair (Cloutier and Castonguay, 1998).

Repair of pyridyloxobutylated DNA was absolutely dependent on ATP. This is consistent with the requirement for ATP during incision and / or oligonucleotide displacement of NER (Wood *et al.*, 1988; Riedl *et al.*, 2003). However, ATP dependence is not exclusive to NER; for example ATP is required for DNA ligation, which is involved in both NER and other DNA repair pathways (Mortusewicz *et al.*, 2006).

The POB adducts 7-POB-dGuo and O^2-POB-dCyd are reportedly non-stable adducts that have the potential to form secondary lesions by rupture of the glycosidic bonds, leading to the formation of AP sites (Lacoste *et al.*, 2006). Although AP sites are

repaired mainly by BER, NER can play a minor role (Frosina *et al.*, 1994; Huang *et al.*, 1994). Therefore it was possible that, in addition to POB adduct repair, a component of the repair activity that was measured in our experimental system was due to repair of AP sites. However, since pyridyloxobutylated plasmid DNA contained a small number of AP sites (0.8 AP site/plasmid) relative to the number of POB adducts (14 adducts/plasmid), it is likely that their repair contributed minimally to the repair activity measured.

Repair of pyridyloxobutylated DNA by normal cell extracts was approximately three times greater than repair of UV damaged DNA, suggesting more efficient repair of pyridyloxobutylated DNA by GGR. Differences in repair efficiency could result from differences in the helix-distorting properties of the two different lesions. Damage recognition is the rate-limiting step in NER (Balajee and Bohr, 2000) and the rate of DNA repair is strongly dependent on the helix distorting properties of the lesion (Hanawalt, 2002). For example, nuclear extracts from human cell lines show differences in repair of UV-light induced DNA lesions versus platinated DNA (Balajee and Bohr, 2000; Hansson and Wood, 1989). As well, differences in DNA repair activities have been shown with structurally different types of UV-light induced lesions, and these differences correlate with adduct cytotoxicity and mutagenicity (Tang *et al.*, 1986).

Consistent with previous findings (Cloutier *et al.*, 2001), NNKOAc-induced single-strand breaks of plasmid DNA. It has been shown that DNA single strand nicks stimulate very little repair incorporation (Wood *et al.*, 1988). Similarly, no significant difference in repair activity was found between nicked and supercoiled damaged DNA

(data not shown), indicating no artifactual increase in damage-specific repair activity due to nicks.

Analysis of the abundance of the four major POB adducts revealed that all the adducts were present at similar levels. This POB adduct profile approximates that which is found in rat lung following *in vivo* NNK treatment (Lao *et al.*, 2006). Relative POB levels observed in this study differ from results obtained previously wherein specific adduct levels varied significantly in calf thymus DNA following *in vitro* treatment with NNKOAc (Sturla *et al.*, 2005; Lao *et al.*, 2006). Differences in DNA adduction could be attributable to structural differences between plasmid DNA and calf thymus DNA. In addition, the ratio of moles of DNA bases to moles of NNKOAc (approximately 1:6) varies from the ratio used with calf thymus DNA (approximately 2:1 and 1:1) (Lao *et al.*, 2006). Although relative levels of specific adducts remained relatively constant when different DNA:NNKOAc ratios were used (Lao *et al.*, 2006), the same ratio used in the present study was not assessed previously and it is possible that the higher amount of NNKOAc used may alter relative POB adduct abundance.

In summary, results presented here are the first to support a role for NER in the repair of NNK-induced pyridyloxobutylation. Insight into the repair processes involved in POB adduct repair is of importance since POB adduct formation is known to play an important role in NNK-induced tumourigenicity in rodents (Staretz *et al.*, 1997a; Trushin *et al.*, 1994; Peterson *et al.*, 2001) and is likely to be involved in tobacco-induced cancers in smokers (Foiles *et al.*, 1991).

CHAPTER 4

IN VIVO TREATMENT WITH 4-(METHYLNITROSAMINO)-1-(3-PYRIDYL)-1-BUTANONE INDUCES ORGAN-SPECIFIC ALTERATIONS IN *IN VITRO* REPAIR OF DNA PYRIDYLOXOBUTYLATION

4.1 INTRODUCTION

NNK selectively induces lung adenocarcinomas in animals (Hecht, 1998) and is believed to be a causal agent in the induction of lung adenocarcinoma in humans, which is now the leading form of human lung cancer (Thun *et al.*, 1997; Hoffmann *et al.*, 1996). However, the mechanisms underlying the inter-organ differences in susceptibility to the carcinogenicity of NNK have not been fully characterized. As anticipated, the extent of both metabolic activation and detoxification of NNK in the lung and liver can affect tumourigenicity (Weng *et al.*, 2007; Hecht, 1998). In addition, DNA repair capacity of the tissue likely contributes to the organoselectivity of NNK (Peterson *et al.*, 2001). Inter-organ differences exist in AGT activity, with higher activity occurring in liver relative to lung. Furthermore, NNK alters activity in an organ-specific manner, significantly decreasing the activity of this repair protein in lung but not in liver (Peterson *et al.*, 2001). However, it is not known if inter-organ differences also exist in NER and whether NNK treatment can alter the activity of this pathway.

NER is mediated by the sequential assembly of repair proteins at the site of DNA damage and involves multiple steps including recognition of the structural distortion and/or chemical alteration in DNA, incision of the damaged DNA strand on either side of the lesion, excision of the damaged oligonucleotide, and resynthesis and ligation of DNA

(Costa *et al.*, 2003). NER can be divided into two subpathways; global genome repair (GGR) which is involved in the removal of lesions from non-transcribed regions of the genome and the non-transcribed strand of transcribed genes, and transcription-coupled repair (TCR) which removes RNA-polymerase-blocking lesions from transcribed strands of active genes (Christmann *et al.*, 2003).

In GGR, the minimum set of NER components necessary for repair reactions consists of XPA, XPC-hHR23B, RPA, XPG, ERCC1-XPF, TFIIH, PCNA, DNA polymerase δ or ε and DNA ligase I (Araujo *et al.*, 2000). XPA, XPC-hHR23B and RPA are necessary for damage recognition and repair protein recruitment; XPG, ERCC1-XPF and TFIIH carry out incision and excision and PCNA; DNA polymerase and DNA ligase I are required for gap-filling and DNA ligation (de Laat *et al.*, 1999).

In the present study, we found that NER activity in mouse lung and liver extracts correlates with previously reported organ-specific susceptibility to NNK-induced carcinogenesis. We also found that *in vivo* treatment of mice with NNK alters *in vitro* repair of NNK-induced DNA adducts, and we examined the mechanism by which these alterations in repair may occur.

4.2 MATERIALS AND METHODS

4.2.1 Animal Treatments

Female A/J mice, aged 9-10 weeks (Taconic Labs, Hudson, NY) were housed with a 12-hour light/dark cycle and provided food and water ad libitum. A/J mice were chosen for this study since NNK carcinogenicity studies have been carried out

predominantly in this strain (Hecht, 1998). Mice were treated with saline (0.1 mL, i.p.) or a single dose of 10 μmol NNK (Toronto Research Chemicals, Toronto, ON) in saline (0.1 mL, i.p.), a dose of NNK that induces lung adenocarcinomas in A/J mice (Hecht *et al.*, 1989). Four or 24 hours following NNK administration, when methyl and POB adducts respectively are maximal (Peterson and Hecht, 1991), mice were killed by cervical dislocation. Lungs and livers were perfused with 10 mM Tris/ 1mM EDTA (pH 7.9), excised, finely chopped, frozen in liquid nitrogen, and stored at –80°C until extract preparation.

4.2.2 Preparation of Cell-Free Whole Tissue Nuclear Proteins Extracts

Nuclear protein extracts from ~ 1.0 to 1.3 g of tissue were prepared from the livers of individual mice and from the pooled lungs of four mice as described (Wood *et al.*, 1995; Wood *et al.*, 1988) with modifications (Bedard *et al.*, 2005). Protein contents of extracts were determined by the method of Lowry *et al.* (1951). Extracts from lung and liver typically contained 6 and 13 mg protein, respectively. Extracts were frozen in liquid nitrogen and stored at –80°C.

4.2.3 Preparation of Pyridyloxobutylated Plasmid DNA

Pyridyloxobutylated plasmid DNA was prepared as described in section 3.2.3

4.2.4 *In Vitro* DNA Repair Synthesis Assay

The repair synthesis assay was performed as described in section 3.2.6 except each 50 μL reaction mixture contained 400 ng of pyridyloxobutylated plasmid DNA or undamaged plasmid DNA, 40 mM Hepes-KOH (pH 7.8), 0.5 mM DTT, 4.0 μmol/L

dTTP, 20 µM of each dGTP, dCTP and dATP, 23 mM phosphocreatine, 18 µg bovine serum albumin (nuclease-free, Sigma), 2.5 µg creatine phosphokinase, 2.0 mM ATP, 5.0 mM $MgCl_2$, 0.4 mM EDTA, 100 mM KCl, 100 µg protein extract and 2.0 µCi [α^{32}P]dTTP (GE Healthcare Amersham). The repair synthesis assay was performed in duplicate for each nuclear protein extract and results are presented as the mean ± SD. To determine damage-specific repair activity, radioisotope incorporation into undamaged DNA was subtracted from repair synthesis activity calculated with POB damaged DNA.

4.2.5 *In Vitro* Incision Assay

The incision assay (Bedard *et al.*, 2005; Coudore *et al.*, 1997; Calsou and Salles, 1994) (Figure 4.1) was performed as described above for the DNA repair synthesis assay (section 4.2.4) except a total reaction volume of 100 µL containing 800 ng of pyridyloxobutylated DNA or undamaged DNA was used in the initial reaction step, from which deoxyribonucleotides were omitted and the DNA polymerase inhibitor, aphidicolin (final concentration, 4.5 µM) was added. After 2 h at 30 ºC, the reaction was terminated by addition of EDTA (final concentration, 25 mM), then treated with 200 µg/mL proteinase K (37 ºC, 30 min) with 0.5 % SDS. Plasmid DNA was purified as for the DNA repair synthesis assay but with gentle mixing to avoid shearing incised DNA. Following extraction, DNA was completely redissolved in TE buffer (10mM Tris/1 mM EDTA (pH 7.9)) by incubation at 30 ºC for 1 h. The redissolved DNA was then incubated in a 100 µL reaction volume containing 90 mM Hepes-KOH (pH 6.6), 2.0 mM DTT, 4.0 µmol/L dTTP, 20 µM of each dGTP, dCTP and dATP, 10 mM $MgCl_2$,

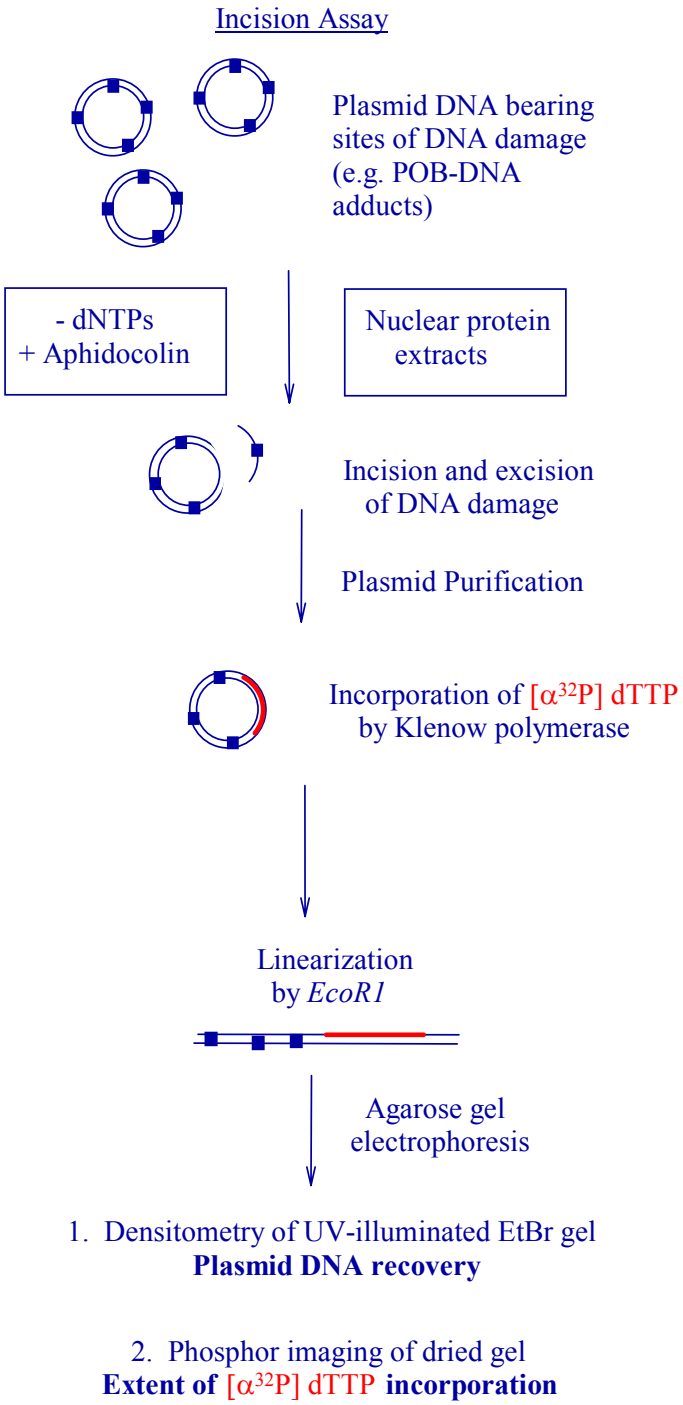

Figure 4.1 Scheme of the *in vitro* incision assay on damaged plasmid DNA by nuclear protein extracts isolated from whole tissue. Squares on plasmid represent DNA lesions (modified from Salles *et al*., 1995a). POB, pyridyloxobutyl; dNTPs, triphosphates of nucleosides; EtBr, ethidium bromide.

2.0 μCi [α^{32}P]dTTP (GE Healthcare Amersham) and 1 U of *E. coli* DNA polymerase I large fragment (New England Biolabs) at 37 ºC for 10 minutes. Samples were incubated for 3 hours at 30°C. Reactions were terminated by adding EDTA to 50 mM and then incubated with 50 μg/mL RNase (37°C for 10 minutes). DNA was purified and then analyzed as described for the DNA repair synthesis assay. The incision assay was done in duplicate for each nuclear protein extract and results are presented as the mean ± SD. To determine damage-specific incision activity, radioisotope incorporation into undamaged DNA was subtracted from incision activity calculated with POB damaged DNA.

4.2.6 Immunoblot Analysis of NER Incision Protein Levels in Mouse Nuclear Protein Extracts

Rabbit polyclonal antibodies anti-human RPA (1/50,000), anti-human XPA (1/50,000), anti-human XPB (1 μg/mL), and anti-human XPC (1/10,000) and chicken polyclonal antibody anti-human GTF2H1 (p62) (1/2,000) and HRP-conjugated goat polyclonal anti-rabbit IgG (1/10,000) and HRP-conjugated goat polyclonal anti-chicken IgY (1/10,000) were purchased from Abcam Inc. (Cambridge, MA). Mouse monoclonal antibody anti-human ERCC1 (1/100), goat polyclonal antibody anti-mouse XPG (1/200) and HRP-conjugated donkey polyclonal anti-goat IgG (1/10,000) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). HRP-conjugated goat polyclonal anti-mouse IgG (1/10,000) was purchased from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA).

Nuclear protein extracts (5-30 µg) were subjected to SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA). Membranes were incubated in 10% milk overnight at 4 ºC and proteins immunoblotted with above antibodies for 1 h at room temperature. After incubating membranes with secondary antibodies conjugated to horseradish peroxidase for 1 h at room temperature, specific proteins were visualized as immunoreactive bands by the enhanced chemiluminescence detection system (Perkin Elmer, Waltham, MA). To normalize for protein loading, total protein was assessed by Amido Black 10B staining of the PVDF membrane. Films and stained blots were scanned and processed with Adobe Photoshop 6.0 Software and densitometry of the scanned image was performed using ImageJ 1.37v Software. Immunoblots were performed in duplicate or triplicate for each nuclear protein extract and results are presented as the mean ± SD.

4.2.7 Analysis of DNA-Associated NER Incision Proteins

An assay to assess the binding of repair proteins to damaged DNA during DNA repair reactions (Figure 4.2) was performed as described (Li *et al.*, 1998; Frit *et al.*, 1998; Salles *et al.*, 1995b) with slight modifications. Briefly, 50 ng of pyridyloxobutylated or undamaged plasmid DNA in 10 mM phosphate buffer (pH 7.0) was adsorbed on Microlite II Microtitre 96 well plates (Thermo Scientific, Waltham, MA) that were pre-treated with 100 µL poly-L-lysine hydrochloride (1 mg/mL in PBS (10 mM phosphate buffer (pH 7.2)/ 150 mM NaCl)) at 30 ºC for 30 min under gentle shaking.

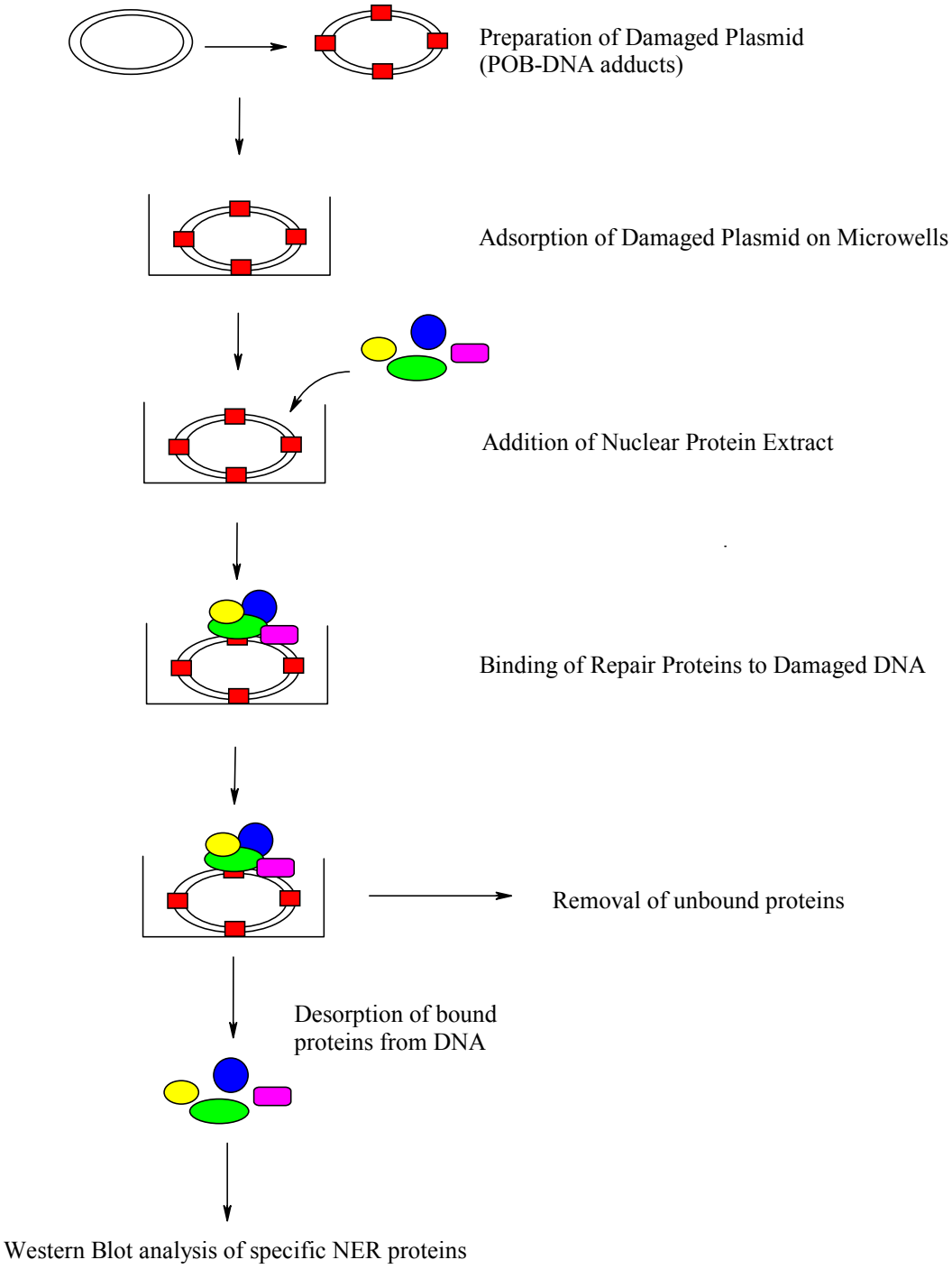

Figure 4.2 Diagramatic representation of the assay used to measure the binding of NER proteins to DNA.

The amount of plasmid DNA adsorbed to wells was verified using PicoGreen (Invitrogen Molecular Probes, Carlsbad, CA). The repair reaction was carried out in 50 µL per well and contained mouse lung (100 µg) or liver (120 µg) nuclear protein extract in 100 mM KCl, 40 mM Hepes-KOH (pH 7.6), 7.0 mM $MgCl_2$, 2.0 mM ATP, 0.5 mM DTT, 10 mM phosphocreatine, 2.5 µg creatine phosphokinase, 2.0 mM EGTA, and 18 µg bovine serum albumin (nuclease-free, Sigma). After 3 h at 30 ºC, wells were washed three times with 50 µL of PBS plus 0.01 % Tween-20, and proteins bound to immobilized DNA were eluted with 25 µL of sample buffer (62.5 mM Tris-HCl (pH 6.8), 4 M urea, 10 % (v/v) glycerol, 2 % (w/v) SDS, 5 % (v/v) β-mercaptoethanol, 0.003 % (w/v) bromophenol blue) for 30 min at 30 ºC with shaking, followed by 15 min at 85 ºC. The proteins were then resolved by SDS-PAGE and analysed as described above for immunoblot analyses except a rabbit polyclonal antibody anti-human XPA (CJ1) generously provided by Drs. C.J. Jones and R.D. Wood was used to assess XPA levels. Each DNA bound protein sample was comprised of reactions pooled from 3 wells. Repair reaction samples were performed in duplicate for each nuclear protein extract.

4.2.8 Data Analysis

Statistically significant differences in repair activity and NER protein levels between treatment groups were determined by unpaired Student's t-tests (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.

4.3 RESULTS

4.3.1 DNA Repair Synthesis and Incision Activities of Mouse Lung and Liver Nuclear Protein Extracts for Pyridyloxobutylated DNA

Mouse liver extracts from saline treated mice exhibited ~3 times greater repair activity towards POB damaged DNA than did those from mouse lung (Figure 4.3a). However, there were no significant differences in the incision of POB adducts between mouse lung and liver extracts (Figure 4.3b).

4.3.2 Effect of *In Vivo* Treatment of Mice with NNK on Repair Synthesis and Incision Activities of Mouse Lung and Liver Nuclear Protein Extracts

Following *in vivo* NNK treatment, repair synthesis activity of mouse lung extracts toward POB damage was decreased by 55 % and 60 % 4 and 24 hours post-dosing, respectively (Figures 4.4a, 4.4b). In contrast, repair activities toward POB-DNA adducts were ~2 and ~3 times higher in liver extracts from NNK treated mice relative to liver extracts from saline treated mice at 4 and 24 hours after treatment, respectively (Figures 4.4c, 4.4d).

To determine if NNK alters repair activity by affecting incision rather than DNA resynthesis, the incision activities of mouse lung extracts from saline or NNK treated mice were compared. NNK treatment had a major effect on incision of POB-DNA adducts in mouse lung extracts, decreasing activity by 92% 4 hours post-dosing (Figure

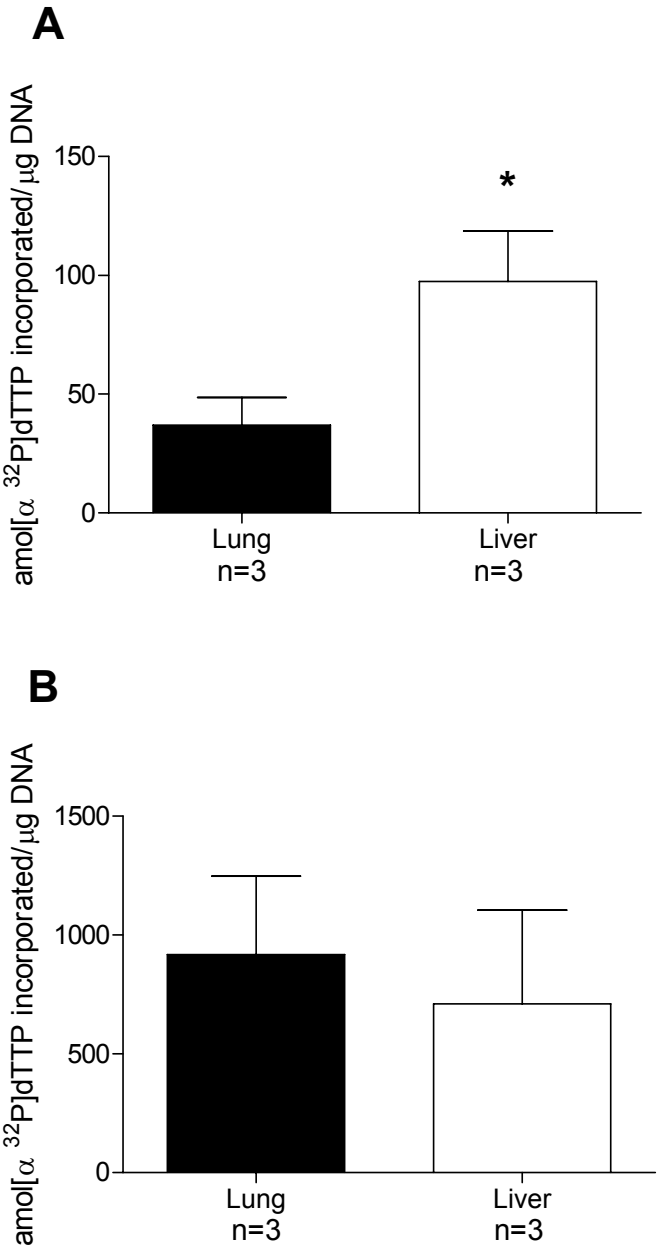

Figure 4.3 *In vitro* DNA repair synthesis (A) and incision (B) of NNK-induced pyridyloxobutyl DNA damage (~14 adducts/plasmid) by nuclear protein extracts isolated from mouse lung (n=3) and liver (n=3) 24 h post-treatment with saline. *, significantly different from lung (P<0.05).

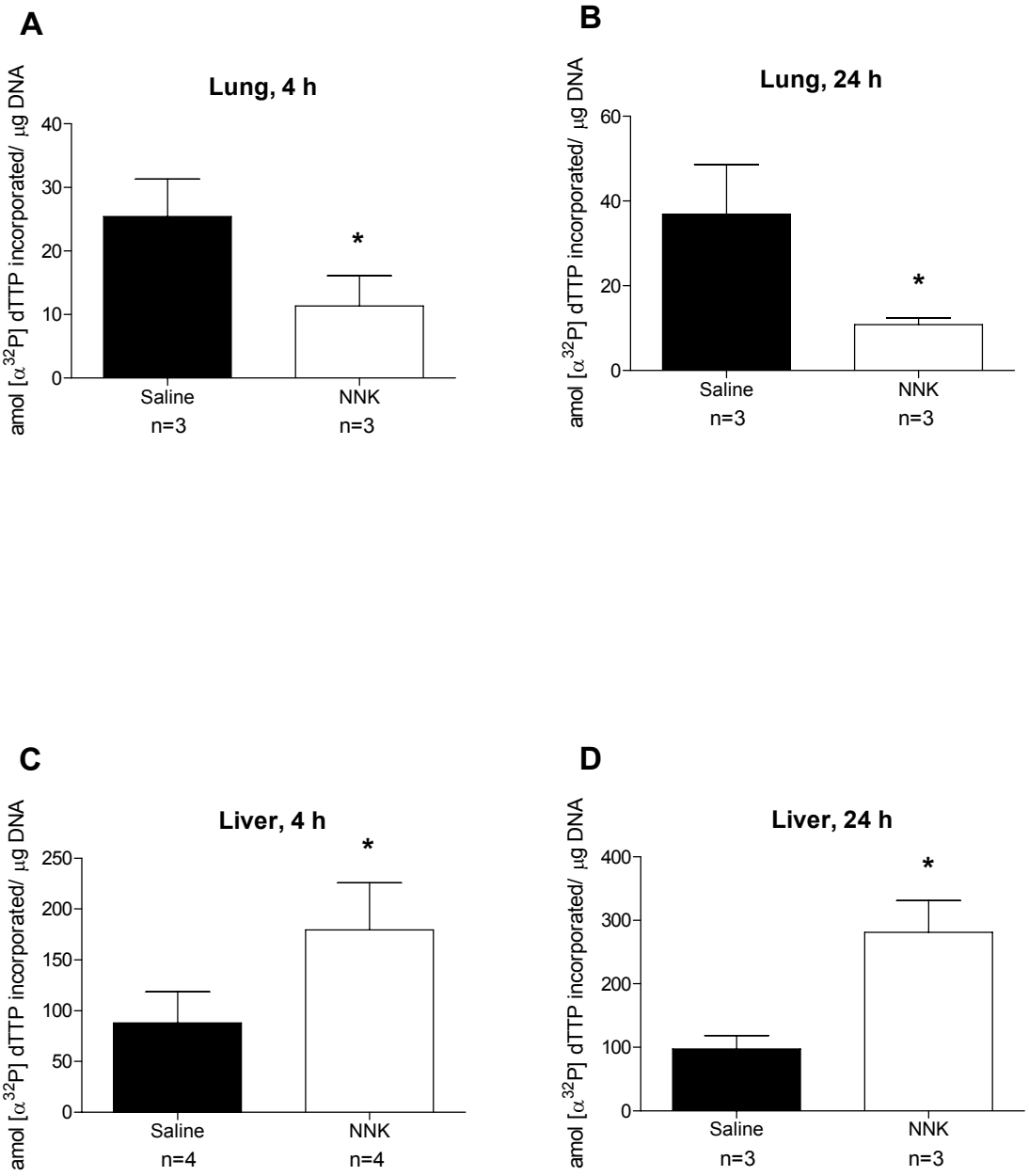

Figure 4.4 Effects of *in vivo* NNK treatment on *in vitro* DNA repair activity of mouse lung (A,B) and liver (C,D) extracts towards NNK-induced pyridyloxobutyl DNA damage. Extracts were prepared from mice treated with either 10 µmol NNK or saline and killed 4 (A,C) or 24 (B,D) h post-dosing. *, significantly different from control (P<0.05).

4.5a). Although an apparent decrease in incision also occurred in mouse lung 24 hours post NNK, this effect did not attain statistical significance (Figure 4.5b). In mouse liver extracts, incision activity was increased by 169% 24 hours post-treatment (Figure 4.5d). Although there was a similar increase in liver extracts from NNK treated mice 4 hours post-treatment, the difference fell just short of statistical significance (P=0.054) (Figure 4.5c).

4.3.3 Effect of *In Vivo* Treatment of Mice with NNK on Levels of NER Incision Proteins in Mouse Lung and Liver Nuclear Protein Extracts

Since NNK significantly altered incision activity, levels of NER incision proteins in mouse lung and liver extracts following NNK treatment were examined (Figure 4.6). At 4 hours post-treatment, NNK only decreased RPA levels in lung extracts by 27% relative to control (Figure 4.7a). In contrast, after 24 hours, NNK increased RPA levels by 43% and increased levels of XPB by 35%, but had no significant effects on the levels of other incision proteins (Figure 4.7b). In mouse liver extracts 4 hours following NNK treatment, there were no significant changes in the levels of any of the incision proteins as compared to liver extracts from saline-treated mice (Figure 4.7c). However, after 24 hours, in liver extracts from NNK treated mice, levels of XPA were 48% higher, levels of XPB were 58% higher and levels of ERCC1 were 209% higher than in liver extracts from control mice (Figure 4.7d).

The possibility existed that significant amounts of repair proteins would be bound to cellular DNA at the time of tissue extract preparation, and this could have affected the

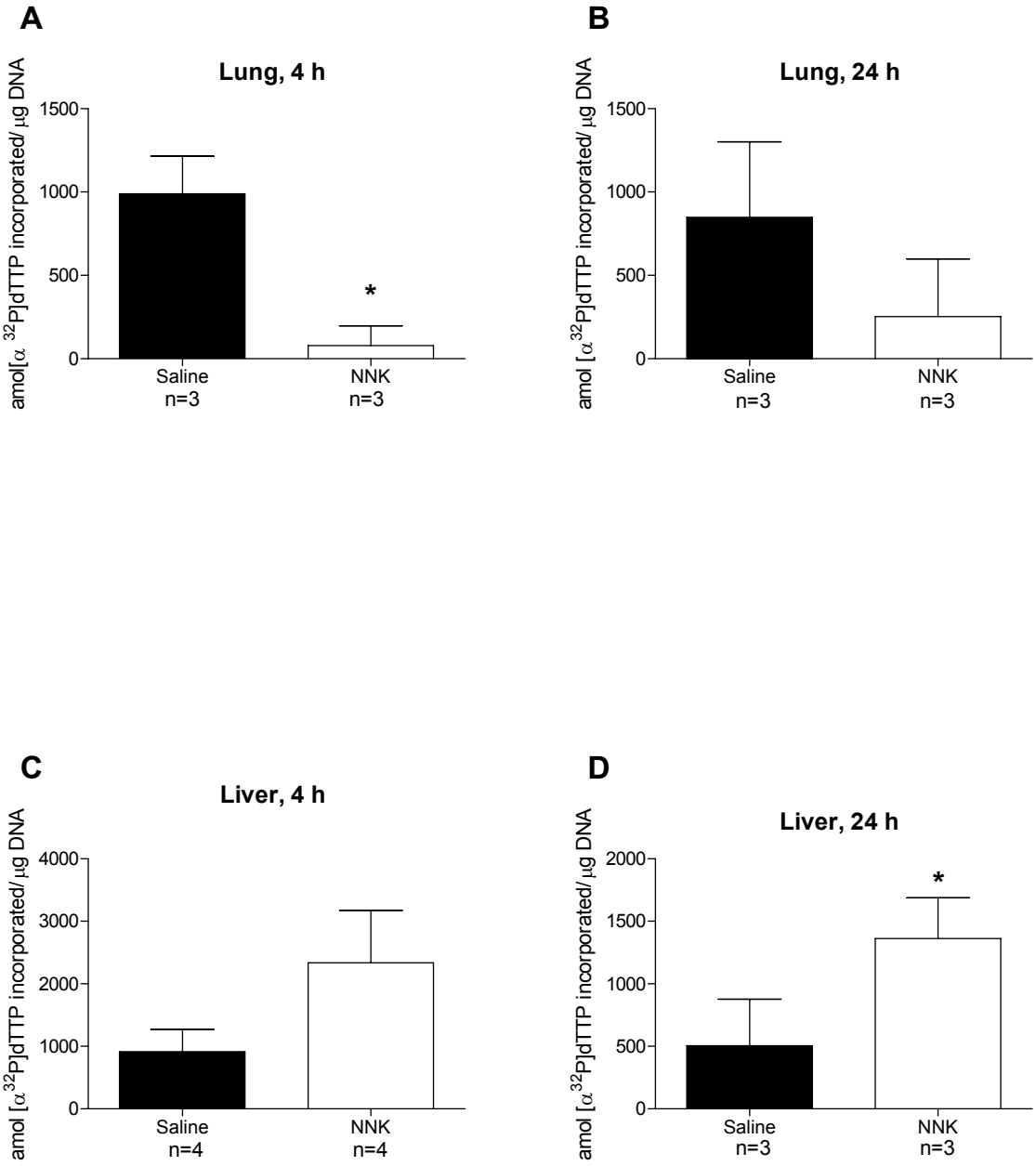

Figure 4.5 Effects of *in vivo* NNK treatment on *in vitro* incision activity of mouse lung (A,B) and liver (C,D) extracts towards NNK-induced pyridyloxobutyl DNA damage. Extracts were prepared from mice treated with either 10 μmol NNK or saline and killed 4 (A,C) or 24 (B,D) h post-dosing. *, significantly different from control (P<0.05).

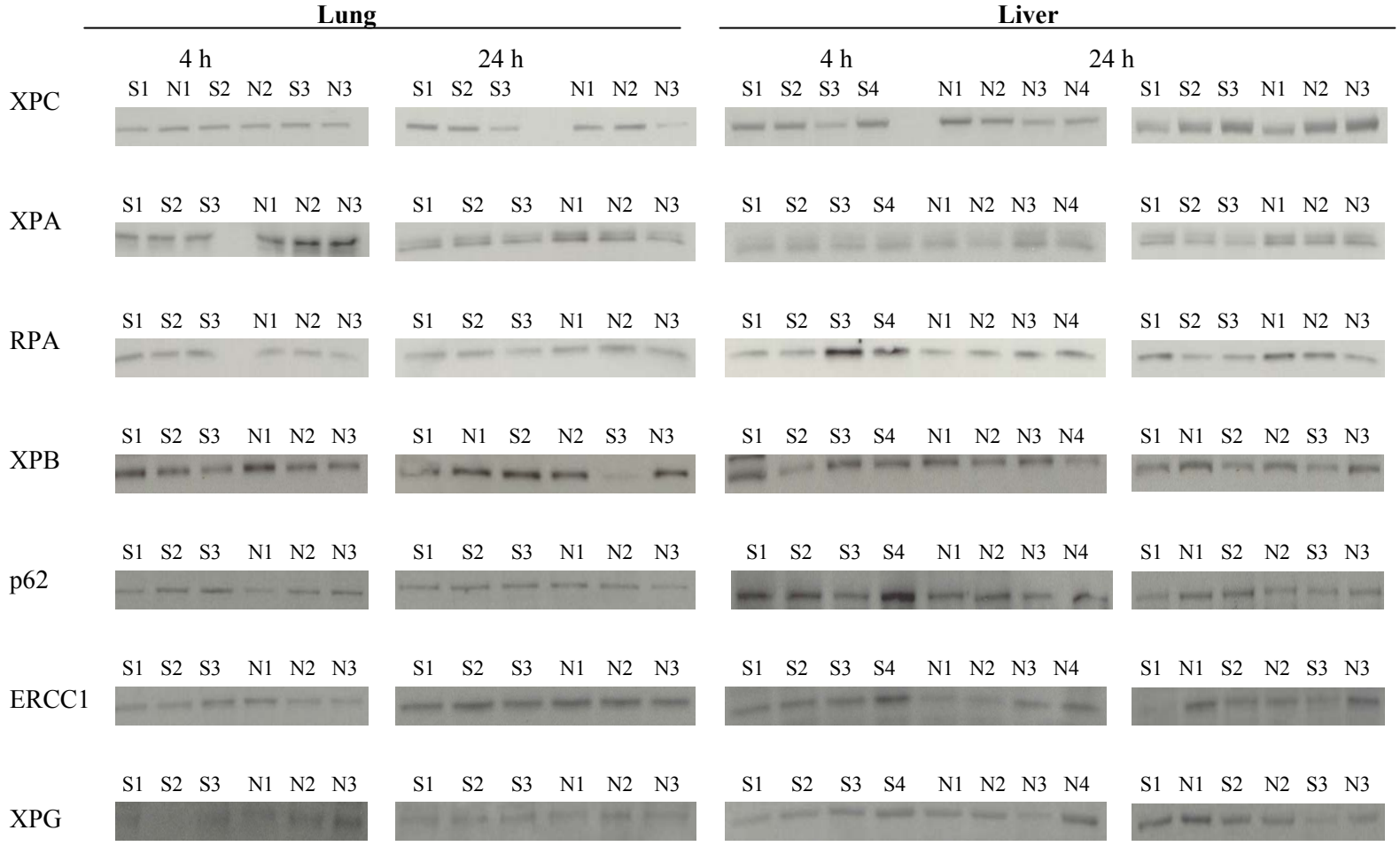

Figure 4.6 Representative immunoblots of the relative levels of each NER protein in mouse lung and liver extracts isolated from saline and NNK-treated mice 4 and 24 h post-dosing. S=nuclear protein extracts from saline treated mice (n=3 for lung (4h, 24h) and liver (24 h) and n=4 for liver (4h)); N=nuclear protein extracts from NNK treated mice (n=3 for lung (4h, 24h) and liver (24h) and n=4 for liver (4h)).

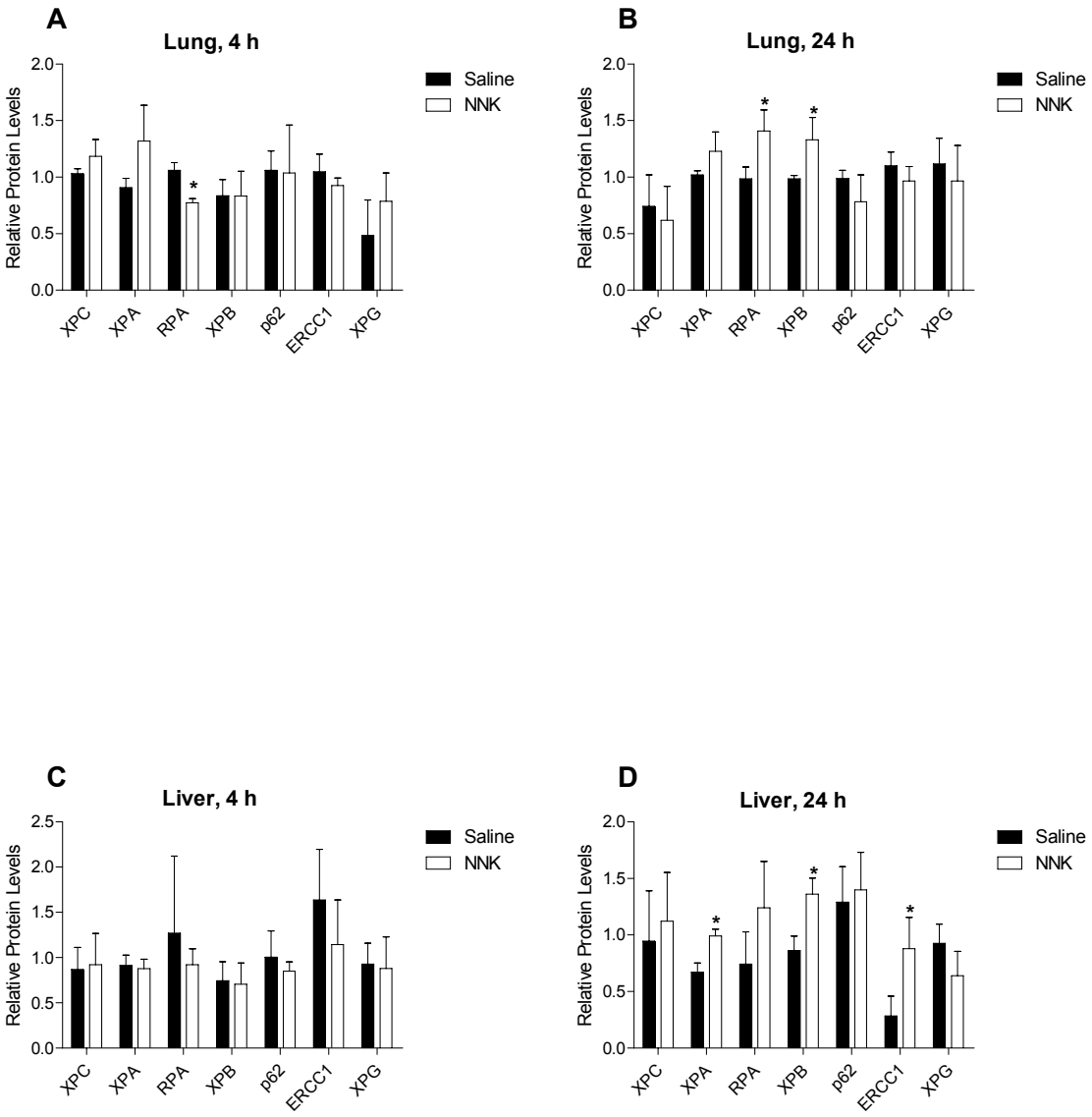

Figure 4 7 Levels of NER proteins in nuclear protein extracts from mouse lung (n=3) (A,B) and mouse liver (n=3) (C,D) at 4 (A,C) and 24 (B,D) h post-treatment with either saline or 10 µmol NNK. Protein levels from immunoblots have been normalized by total amount of protein based on Amido Black staining. *, significantly different from control (P<0.05).

recovery of repair proteins in the DNA-free fractions used for repair assays (i.e. "nuclear protein extract fractions"). To examine this possibility, immunoblots of XPA, RPA, XPB, and ERCC1 were assessed in the DNA-containing fractions. There was no detectable XPB or ERCC1 in the DNA-containing fractions (lower limit of detection < 5% of that detected in nuclear protein extract fractions). Both XPA and RPA were detectable in the DNA-containing fractions, but no differences occurred between samples from saline- and NNK-treated mice (data not shown). This confirmed that treatment-related effects on levels of repair proteins and repair activities were not attributable to differences in recovery.

4.3.4 Effect of *In Vivo* Treatment of Mice with NNK on NER Protein DNA Binding in Mouse Lung and Liver Nuclear Protein Extracts

To determine if NNK was affecting the interactions between repair proteins and damaged DNA, an assay was used that measures the binding of repair proteins to damaged DNA during the NER reaction (Frit *et al.*, 1998; Li *et al.*, 1998; Salles *et al.*, 1995b).

Binding of XPA and XPB to POB damaged DNA was decreased in mouse lung extracts both 4 and 24 hours following NNK treatment (Figure 4.8) even though levels of both immunoreactive proteins in extracts were similar between treatment groups. Binding of XPA and XPB to POB damaged DNA was increased in mouse liver extracts 4 and 24 hours after NNK treatment (Figure 4.8).

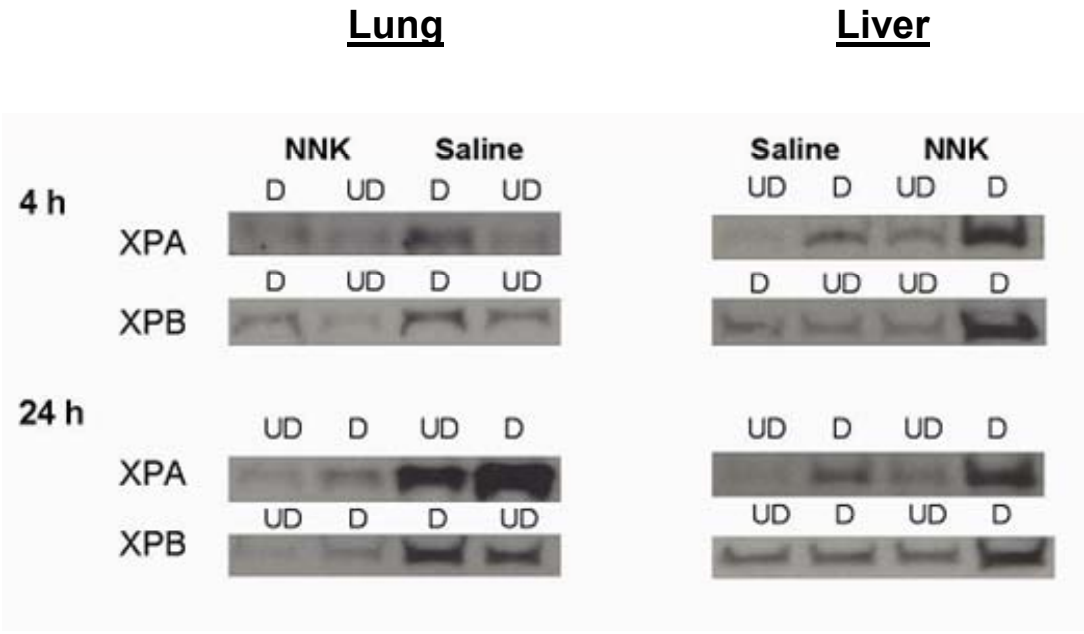

Figure 4.8 Effects of *in vivo* NNK treatment on binding of XPA and XPB from mouse lung and liver nuclear extracts to pyridyloxobutyl damaged DNA. Lung and liver extracts were prepared from mice treated with either 10 μmol NNK or saline and killed 4 or 24 h post-dosing. XPA and XPB binding to DNA was determined by Western blotting of the protein fraction retained on DNA in the microplate wells. UD, undamaged DNA; D, POB damaged DNA. Blots shown are representative of results obtained from experiments with lung (n=3) and liver (n=4) extracts.

4.4 DISCUSSION

This is the first study to demonstrate that NNK alters repair of POB-DNA adducts by altering of the NER process. NNK treatment inhibits repair of POB-DNA adducts in mouse lung extracts and increases repair in mouse liver extracts, and these effects are associated with perturbations of key incision repair proteins. Our findings suggest that both differences in basal DNA repair activity and NNK-mediated changes in repair contribute to the organ-selective carcinogenicity of this nitrosamine in the mouse. Interestingly, similar effects occurred with another mouse lung carcinogen, the mycotoxin aflatoxin B_1 (AFB_1) (Bedard *et al.*, 2005), suggesting that alteration of DNA repair may be an important mechanism underlying organ-specific susceptibility to numerous carcinogens. Moreover, the ability of some carcinogens to not only cause DNA damage, but also to alter repair of damage caused either by themselves or by other genotoxic agents, may be a frequent but unappreciated property of these agents. Indeed, although pyridyloxobutylating compounds are relatively weak lung carcinogens in the A/J mouse (Peterson and Hecht, 1991), they increase the tumourigenicity of methylating agents, both by producing adducts and by inhibiting lung AGT activity, leading to increased persistence of O^6-mG (Peterson *et al.*, 1993; Peterson *et al.*, 2001). Therefore, inhibition of POB adduct repair by the NER pathway in lung would impact on the persistence of both POB adducts and O^6-mG adducts, both of which contribute to NNK carcinogenicity (Staretz *et al.*, 1997a; Peterson and Hecht, 1991; Trushin *et al.*, 1994). Perturbation of DNA repair by NNK may also extend beyond effects on NER and direct damage reversal by AGT. For example, there is *in vitro* evidence that NNK activates the

oncogenic proteins Bcl-2 and c-Myc, which repress repair of abasic sites by base excision repair (Jin *et al.*, 2006).

It is also noteworthy that alteration of NER is not limited to established genotoxic agents; although *in vivo* data are limited, inflammatory mediators (Gungor *et al.*, 2007), arsenic(Shen *et al.*, 2008), nickel (Hu *et al.*, 2004) and cadmium (Fatur *et al.*, 2003) all inhibit NER in cultured cells. The fact that NNK's effects on overall repair synthesis activity coincide with alterations in the incision phase, is consistent with decreased levels of RPA. Along with XPA, TFIIH and XPC, RPA is necessary to form the preincision complex of NER at sites of DNA damage (Li *et al.*, 1998). Specifically, the RPA-XPA complex is a key component of damage recognition, and RPA complexed with XPA displays greater affinity for damaged DNA than either RPA or XPA alone (Patrick and Turchi, 2002). Therefore, a decrease in RPA levels may have contributed to the decrease in DNA binding of XPA and XPB observed 4 hours post NNK. However, since NNK increased RPA and XPB levels at 24 hours, alterations in the levels of RPA cannot explain the observed inhibition of repair in mouse lung at the later timepoint.

Decreased binding of XPA to DNA in mouse lung following NNK treatment is of particular interest. XPA has no catalytic activity, but rather is a core structural factor that interacts with DNA as well as RPA, TFIIH and ERCC1, which are factors in the preincision complex of NER. In the absence of XPA, the requisite stable preincision complex cannot form and NER cannot occur (Koberle *et al.*, 2006). In fact, anti-XPA antibodies that hinder XPA binding to RPA, ERCC1 and TFIIH, inhibit NER reactions (Saijo *et al.*, 2004). Therefore, perturbation of XPA by NNK, which interferes with XPA

protein-protein interactions, could be responsible for the decrease in repair activities in lung. This is consistent with the fact that NNK also caused decreased binding of XPB (a component of TFIIH) to DNA.

In contrast to the effects of NNK in lung, repair and incision activities in liver were increased 4 and 24 hours following NNK treatment. Induction of repair activity is well established in bacteria as a part of the SOS response (Foster, 2007) and more recently, UV irradiation and AFB_1 treatment have been shown to enhance NER both in mammalian cells (Protic *et al.*, 1988; Germanier *et al.*, 2000) and in nuclear protein extracts prepared from tissues of animals treated *in vivo* (Bedard *et al.*, 2005).

The persistence of elevated repair and incision activities in liver from 4 to at least 24 hours suggests a disturbance in the expression of repair enzymes. While there were no significant alterations in liver incision protein levels 4 hours after NNK treatment, levels of XPA, XPB and ERCC1 were increased at 24 hours. Increases in XPA and XPB proteins were consistent with the increases in binding of each protein to POB damaged DNA; it has been demonstrated that the cellular concentration of XPA significantly affects repair activity (Koberle *et al.*, 1999; Koberle *et al.*, 2006); XPA binds more efficiently to DNA adducts as a dimer than as a monomer, and dimer formation is concentration-dependent (Hernandez-Ruiz *et al.*, 2001). Moreover, over-expression of XPA increases repair activity and survival of cells following UV irradiation (Cleaver *et al.*, 1995). Similarly, over-expression of XPB is linked to increased DNA repair (Vogel *et al.*, 2000).

Attempts were made to assess both the levels and DNA binding activities of all key NER incision proteins. However, due to the unavailability of antibodies with adequate specificity and sensitivity, levels of XPF and XPD could not be determined, and thus effects of NNK on repair activity could also be due in part to changes in those proteins. Also, because of limited antibody sensitivity, only the binding of XPA and XPB could be assessed and DNA binding could only be evaluated qualitatively since the desorption of repair proteins bound to DNA may not be reliably quantitative (Li *et al.*, 1998). Assessment of the binding of other incision proteins, notably XPC, would be of interest since both XPC and XPA are necessary for damage recognition, the rate-limiting step of the NER process, and for the early stages of repair protein recruitment (Balajee and Bohr, 2000; Volker *et al.*, 2001).

The fact that increases in repair activities in liver and decreases in lung were not always accompanied by corresponding increases or decreases in incision protein levels at both timepoints examined, suggests that either the mechanisms responsible for the changes in repair activities are different at 4 and 24 hours post-treatment, or that NNK may be mediating its effects on repair by altering proteins that are not directly involved in the incision step of NER. For example, the tumour suppressor proteins p53 and BRCA1 play roles in regulating GGR through transcriptional induction and/or recruitment of repair proteins (Wang *et al.*, 2003c; Chang *et al.*, 2008; Hartman and Ford, 2002), so changes in these proteins could affect incision proteins and contribute to the observed effects on repair.

The inter-organ differences in repair activity observed in control mice are likely attributable to differences in the post-incision phase of NER since there were no significant differences in incision activities of extracts between lungs and livers. In fact, DNA polymerases may account for differences in DNA repair synthesis since addition of the Klenow fragment (from *E. coli* DNA polymerase I) eliminated the differences in repair between lung and liver.

The relevance of our findings to other species susceptible to NNK-induced carcinogenesis is not known. In A/J mice, NNK is mainly a lung carcinogen (Hecht, 1998) but in rats, NNK is carcinogenic in the lung, liver and other sites (Hecht, 1998), suggesting that if NNK affects NER in the rat, it may have different inter-organ effects compared to the mouse.

In summary, results from this study demonstrate that *in vivo* treatment with NNK alters DNA repair synthesis and incision activities in mouse lung and liver, and these alterations coincide with inter-organ differences in susceptibility to NNK. NNK alters both the damage recognition and post-recognition steps that control incision/excision by altering the binding of XPA and XPB to damaged DNA. The fact that changes in levels of some repair proteins did not correspond with changes in repair activity suggests that NNK may also alter levels of proteins that are not directly involved in the repair process and / or that NNK produces its effects through covalent modification of proteins. Insight into the repair processes involved in POB adduct repair is of importance since POB adduct formation plays an important role in NNK-induced tumourigenicity in rodents

(Staretz *et al.*, 1997a; Trushin *et al.*, 1994; Peterson *et al.*, 2001) and is likely to be involved in tobacco-induced cancers in smokers (Foiles *et al.*, 1991).

CHAPTER 5
DISCUSSION

5.1 GENERAL DISCUSSION

The studies described in this thesis were directed at investigating the molecular mechanisms involved in the induction of pulmonary carcinogenesis by the tobacco-specific nitrosamine, NNK, by addressing two fundamental determinants of mutagenicity; biotransformation and DNA repair.

NNK requires bioactivation to exert its carcinogenicity and cytochromes P450 are thought to be the major catalysts of NNK biotransformation. Although CYP2A6/2A13, CYP2B6, CYP3A4/3A5 and CYP2E1 (Smith *et al.*, 1992a; Smith *et al.*, 1995; Hecht, 1998; Smith *et al.*, 1999; Smith *et al.*, 2003) have all been implicated in human pulmonary NNK metabolism, identification of the major CYP isozyme(s) that are responsible remains to be elucidated. Determination of the major CYPs involved in NNK bioactivation and detoxification may lead to a better understanding of smoking-induced lung cancer etiology and to the development of novel prevention and therapeutic strategies. Because of their high metabolic activity for NNK bioactivation and their substantial expression in human lung, the CYP2A enzymes, CYP2A13 and CYP2A6 are thought to contribute largely to human pulmonary NNK metabolism. When examining lung microsomal NNK metabolism data from 29 individuals, no significant correlations were found between measures of CYP2A activity or expression and the degree of total NNK bioactivation or detoxification. However, subgroups of individuals were identified

for whom CYP2A13 mRNA correlated with total NNK and NNAL α-hydroxylation as well as NNAL-*N*-oxide formation. In addition, the degree of NNAL formation and CYP2A13 mRNA were also correlated (Chapter 2). The finding that high CYP2A13 expression correlated with NNK bioactivation in a subset of individuals is consistent with results simultaneously published by another group (Zhang *et al.*, 2007). While our results do not suggest uniform involvement of CYP2A13 in NNK metabolism across all individuals, the possibility exists that more sensitive and specific measures (ability to detect low CYP protein levels, highly specific substrate or inhibitor for CYP2A6 or CYP2A13) of enzyme contributions would reveal that a correlation exists for a greater proportion of individuals. The fact that individuals could be classified as either high or low bioactivators and detoxifiers reflects the existence of sub-populations with differences in abilities to convert NNK to DNA-damaging species or convert NNK to excretable detoxified products. While these differences were not attributable to current smoking habits, gender, or age, if the basis for these differences could be determined, identification of high and low categories for bioactivation and detoxification may be useful for predicting susceptibility of individuals to NNK-induced carcinogenesis.

Results presented in this thesis are the first to demonstrate a role for NER in the repair of NNK-induced pyridyloxobutylation; NER-deficient XP cells, XPA and XPC, were less active at repairing POB adducts than were extracts from normal cells, while combining NER-deficient extracts reconstituted activity. Also, NER-deficient cells were more susceptible to NNKOAc-induced cytotoxicity than were normal cells (Chapter 3). Elucidation of the repair processes involved in POB adduct repair is of importance since

POB adduct formation is known to play an important role in NNK-induced tumourigenicity in rodents (Staretz *et al.*, 1997a; Trushin *et al.*, 1994; Peterson *et al.*, 2001) and is likely to be involved in tobacco-induced cancers in smokers (Foiles *et al.*, 1991). Moreover, characterization of POB-DNA adduct repair pathways may allow for the identification of susceptible individuals based on their ability to repair POB-DNA damage. Thus, individuals with repair pathway deficiencies due to genetic polymorphisms in repair proteins and/or environmental exposures may have a higher risk for the development of tobacco-related cancers. In addition, determining the involvement of specific repair pathways could also be useful in identifying potential targets for chemoprevention therapies.

NNK has been established as a potent and highly selective inducer of lung carcinogenesis in animals and is believed to be a causal agent in the induction of human lung adenocarcinoma (Thun *et al.*, 1997; Hoffmann *et al.*, 1996). The mechanisms underlying the susceptibility of lung to NNK carcinogenicity have not been fully characterized. Elucidation of these mechanisms underlying target organ-specific susceptibility to NNK and other carcinogenic chemicals will be beneficial in estimating the risk that these chemicals pose to humans and in explaining and predicting interindividual differences in susceptibility. While there is evidence that NNK biotransformation is an important factor contributing to the organoselectivity of NNK (Hecht, 1998; Weng *et al.*, 2007), results demonstrated in this thesis also support a role for DNA repair in the mouse model (Chapter 4). Mouse lung had significantly lower DNA repair activity for POB DNA-adducts as compared to liver and *in vivo* treatment

with NNK decreased NER activity in lung but increased NER activity in liver. While the precise mechanisms responsible for the alterations in repair could not be fully elucidated, our findings demonstrated that NNK targets the incision phase of NER, specifically both the damage recognition and post-recognition steps involved in controlling incision/excision by altering the binding of XPA and XPB to damaged DNA. Moreover, the alterations in repair activity that coincide with organ-specific differences in susceptibility to NNK may be a key mechanism underlying the susceptibility of lung to NNK-induced carcinogenesis. Also, it is possible that carcinogen-induced alterations in repair are not limited to a small number of chemicals but rather may be a fundamental mechanism underlying target-organ susceptibility to many cancer-causing chemicals. From a therapeutic perspective, induction of repair activity and hence an enhanced ability to deal with DNA damage may be beneficial for the human population in general, and particularly for those people with repair deficiencies.

5.2 FUTURE DIRECTIONS

5.2.1 Specific cytochrome P450 isozymes are important in NNK biotransformation and differences in the levels of these isozymes are associated with epigenetic factors.

The results from chapter 2 demonstrate that while CYP2A13 is not a major contributor to NNK bioactivation in all individuals, it seems to play a role in some. It is possible that more sensitive measurements of CYP proteins would allow for a better assessment of the enzymes involved. In addition to the CYP2A proteins, it is possible that that several different human pulmonary CYPs or combinations of CYPs are involved in NNK metabolism (Table 1.1). Recently, an extremely sensitive and highly specific

liquid chromatography tandem mass spectrometry (LC-MS/MS) method for quantifying proteins has been developed which probably could be applied to the quantification of CYPs in human lung microsomes, where multiple CYPs are expressed at very low levels (Kuhn *et al.*, 2004; Anderson and Hunter, 2006). Using this approach, all CYPs implicated in NNK metabolism could be quantified and if a single CYP or a combination of CYPs is primarily responsible for NNK metabolism, then its/their levels should correlate with NNK metabolism.

Since CYPs are thought to be the major contributors to NNK bioactivation and detoxification, it is expected that the relative expression of specific CYPs is probably responsible for the dichotomies observed (Chapter 2). Thus, determining the levels of different CYPs will also help in the identification of CYPs that are expressed at significantly different levels between high and low bioactivators and detoxifiers. Since the distribution of subjects between bioactivation categories is not consistent with reported frequencies of established genetic polymorphisms of enzymes implicated in NNK metabolism, it is likely that epigenetic factors, rather than genetics, make the largest contribution to the dichotomies in NNK metabolism.

Numerous epigenetic factors contribute to altered CYP expression. Disease states such as inflammation affect the levels of CYPs in the lung and using a mouse model of inflammation, levels of CYP2E1 and CYP2F1 have been found to be altered (Stoilov *et al.*, 2006). More recently, studies have begun to examine the role of promoter methylation, histone acetylation status and microRNA in the regulation of CYP expression. The level of methylation in the promoter/enhancer regions of genes encoding

for CYP1A1, 1A2 and 1B1 influences the mRNA and protein levels of each of these proteins (Tsuchiya *et al.*, 2006; Anttila *et al.*, 2003). Moreover, inhibition of histone deacetylase and DNA methylase increases levels of the CYP1A proteins (Nakajima *et al.*, 2003; Tokizane *et al.*, 2005), while DNA methylation suppresses TCDD-induced expression of CYP1A1 (Okino *et al.*, 2006). Both promoter methylation and histone acetylation also regulate CYP2A13 expression (Ling *et al.*, 2007). MicroRNA regulates levels of CYP1B1 post-transcriptionally (Tsuchiya *et al.*, 2006) and activation of the AhR receptor by cigarette-smoke constituents positively regulates CYP1A1 and CYP1A2 (Okey, 2007; Harper *et al.*, 2006). All of these parameters can be assessed experimentally and potential associations with CYP levels and the degree of NNK metabolism can be carried out. Identification of the epigenetic factors contributing to differences in expression of the major CYPs involved in NNK metabolism may point to specific factors that could be manipulated by chemopreventive agents that alter the extent of NNK bioactivation or detoxification in smokers, and thus lower lung cancer risk in exposed individuals.

5.2.2 Alterations in DNA repair activity in mouse lung and liver following *in vivo* treatment with NNK involved covalent modification of key NER incision proteins and/or alterations in the levels and/or activities of proteins not directly involved in the NER reaction.

Although the results outlined in chapter 4 demonstrate that *in vivo* treatment with NNK alters repair activity and incision activity specifically, in mouse lung and in mouse liver, the precise mechanisms responsible for the alterations in repair are unknown.

Although NNK altered the binding of XPA and XPB to damaged DNA, these differences could not be fully explained by changes in the levels of key incision proteins, suggesting that NNK may alter protein activity not by changing the levels of incision proteins but rather by direct protein modification such as the formation of covalent protein adducts. HPB-releasing hemoglobin adducts have been detected in rats following NNK treatment and in smokers (Hecht and Trushin, 1988; Hecht *et al.*, 1994), but it is not known if NNK also forms adducts with NER proteins. Potentially adducted NER proteins such as XPA and XPB can be separated by SDS-PAGE and, following immunoblotting, excised, digested and subjected to mass spectrometry analysis. Proteomics (including immunological or enzymatic techniques as well as mass spectrometric analysis) could also be used to determine if NNK is altering protein activity by changing the post-translational modification of NER proteins. Moreover, it is known that phosphorylation of a serine residue of XPB inhibits NER activity by preventing XPF-ERRC1-mediated incision (Coin *et al.*, 2004), and while phosphorylation of both RPA and XPA can occur, this modification either has no effect on NER activity (the case for RPA) or the effects on NER activity are not known (the case for XPA) (Wu *et al.*, 2006; Ariza *et al.*, 1996). In addition, recently, XPA-dependent sumoylation of XPC has been demonstrated and may be important in XPC stabilization, protein-protein interactions, or activity (Wang *et al.*, 2005a). Since many of these protein modifications are associated with changes in NER activity, determining which NER proteins are affected by NNK treatment will provide valuable mechanistic data as to which of the NER proteins are essential for maintaining

the activity of the NER pathway as well as which proteins can be manipulated therapeutically to alter the carcinogenic effects of NNK in smokers.

In addition to modifying the NER proteins directly involved in the steps of NER, it is possible that NNK is mediating its effects on repair by altering the function and/or levels of proteins that are not directly involved. One such example could be p53. p53 plays a key role in regulating GGR in response to DNA damage through transcriptional induction and/or recruitment of repair proteins such as XPC, DDB2 (p48) (Wang *et al.*, 2003c), XPB (Chang *et al.*, 2008), TFIIH and RPA (Wang *et al.*, 2003c). p53 influences the rate of repair of DNA pyridyloxobutylation damage (Lacoste *et al.*, 2007) and NNK treatment increases the expression of p53-responsive genes (Ellinger-Ziegelbauer *et al.*, 2004; Lonardo *et al.*, 2002). Although some studies have shown that p53 failed to influence NER *in vitro* (Wang *et al.*, 2003c), a recent study reported that p53 was vital to the *in vitro* repair activity of nuclear extracts (Chang *et al.*, 2008). Thus, p53 or a lack thereof may be influencing repair following NNK treatment by modifying levels of other repair proteins not assessed in this study such as DDB2, or by altering the recruitment of XPC and/or XPB to damaged DNA. Immunoblotting could be used to determine if NNK treatment changes p53 protein levels in mouse extracts and p53 reconstitution experiments could be performed to determine if lack of p53 alters the recruitment of essential incision proteins to damaged DNA. Also, immunological techniques could be carried out to determine if NNK treatment alters p53 protein interactions with XPC or XPB and since post-translational modifications of p53 are critical in regulation of p53

stability and activity (Appella and Anderson, 2001; Ito *et al.*, 2001; Gostissa *et al.*, 1999), the effects of NNK on these processes could also be examined using proteomic methods.

5.2.3 Inhibition of incision activity following *in vivo* treatment with NNK varies between different mouse lung cell types.

Although results from chapter 4 demonstrate that NNK inhibits incision activity in whole mouse lung, it is not known whether these effects are concentrated in putative target lung cell types. In rat lung, the levels of HPB-releasing POB-DNA adducts in alveolar type II cells correlate with lung tumour incidence over a range of NNK doses (Belinsky *et al.*, 1990). However, levels of O^6-mG in Clara cells also correlate with lung tumour incidence despite the fact that type II cells and not Clara cells are believed by some investigators to be the cells of origin of the tumours in rats (Belinsky *et al.*, 1990). Similarly, in A/J mice, type II cells are postulated to be the precursor cells for NNK-induced tumours and a role for alveolar type II cells in NNK activation and tumourigenesis has been demonstrated (Belinsky *et al.*, 1991; Belinsky *et al.*, 1992). Moreover, it is possible that differences in DNA repair activity between these cells types could contribute to the relative susceptibility of these cells to NNK carcinogenesis.

Low level repair of O^6-mG occurs in Clara cells, while higher levels of repair are found in type II cells and macrophages (Belinsky *et al.*, 1991; Belinsky *et al.*, 1988). Following NNK treatment, AGT activity is not affected in macrophages while significant decreases in repair are observed in type II cells and Clara cells (Belinsky *et al.*, 1988). Conversely, there is no information about NER activity or POB adduct repair in different mouse lung cell types.

Using the methodology from Chapters 3 to prepare nuclear protein extracts from freshly isolated lung cell types is unrealistic since the numbers of cells required for the preparation are significantly higher than what could reasonably be obtained from isolated mouse lung tissue. Using a modified comet assay approach (an assay that measures DNA damage by assessing DNA strand breaks in electrophoresed DNA from individuals cells) to assess repair, protein extracts could be prepared from a smaller number of cells and incision activity of extracts from those cells measured by adding extracts to NNKOAC-exposed gel embedded cellular DNA and determining the extent of nicked DNA by fluorescent staining and microscopy (Langie *et al.*, 2006). Thus, extracts with higher repair (incision) activity would produce a greater degree of nicked DNA. Even though this modified repair assay only measures incision activity, the incision phase of NER is inhibited by NNK in mouse lung**.** If previous observations regarding relative susceptibility of specific cell types are related to low DNA incision activity and/or to its susceptibility to inhibition by NNK, this should be reflected by treatment effects and cell type differences in incision activity. Thus, assessing differences in DNA adduct repair and the effects of NNK treatment on DNA repair among different cell types will potentially demonstrate a role for DNA repair in the relative susceptibility of type II cells to NNK carcinogenicity and may help establish a basis for identifying tumour cell progenitors.

5.2.4 DNA repair in human lung tissues is affected by cigarette smoking status and by genetic polymorphisms in repair genes.

DNA repair studies outlined in chapter 4 have assessed the effects of a single carcinogen, NNK, on DNA repair activity and have used the mouse model. It is of interest to determine if these findings can be extrapolated to humans using tobacco smoke, a complex mixture of carcinogens. This will provide information as to the relevance of the mouse model for assessing carcinogen-induced alterations in DNA repair activity as well as valuable mechanistic information regarding the importance of DNA repair in lung cancer development in humans.

DNA repair activity probably contributes substantially to lung cancer risk (Wei *et al.*, 2000; Wei and Spitz, 1997; Spitz *et al.*, 2003; Paz-Elizur *et al.*, 2003). However, studies assessing NER activity in humans have been limited to lymphocytes (Wei *et al.*, 2000; Wei and Spitz, 1997; Spitz *et al.*, 2003), so little is known about how well bulky DNA adducts are repaired in human lung, or if repair is affected by either smoking or polymorphisms in NER genes. The findings in chapter 4 demonstrate that NNK inhibits POB adduct repair in mouse lung, and inflammatory mediators from cigarette smoke inhibit NER in human cultured pulmonary epithelial cells (Gungor *et al.*, 2007), so it is possible that smoking may alter POB-DNA adduct repair in human lung. To assess if smoking alters repair in human lung, methodology outlined in chapter 4 can be used to prepare nuclear protein extracts from human lung specimens (tissue obtained as in Chapter 2) and to assess DNA repair activities of POB adducts. Associations between reported smoking histories from individuals and DNA repair activity can be examined to

determine the effects of smoking on NER activity. DNA repair activity by human lung extracts will also likely be influenced by genetically determined polymorphisms in genes that encode NER proteins. Moreover, polymorphisms in key NER proteins have been observed and many of these are associated with effects on DNA repair activity and alterations in human respiratory cancer risk (Wang *et al.*, 2007b; Sturgis *et al.*, 2002; Vodicka *et al.*, 2004; Vodicka *et al.*, 2007; Marin *et al.*, 2004; Wu *et al.*, 2003). Individuals can be genotyped using methodology described in Chapter 2 and associations with variant alleles in NER genes can be made with DNA repair activity to determine the influence of the polymorphisms on repair.

POB-DNA adducts have been measured in lung tissue from smokers (Foiles *et al.*, 1991) and individual POB-DNA adducts, O^2-POB-dThd, O^6-POB-dGuo, 7-POB-dGuo and O^2-POB-dCyt have been quantified in rat lung following NNK treatment (Lao *et al.*, 2006; Lao *et al.*, 2007). However, there is no information on the relative efficiencies by which these adducts are repaired in human lung. The pyridyloxobutylated plasmid DNA described in Chapter 3, which contains similar levels of the four major POB-DNA adducts, O^2-POB-dThd, O^6-POB-dGuo, 7-POB-dGuo and O^2-POB-dCyt, will be an ideal substrate to measure relative adduct repair in nuclear extracts prepared from human lung. Mass spectrometric analysis (Chapter 3) can be used to measure levels of POB-DNA adduct before and after repair by human lung extracts. Recently, in rat lung, the levels of individual POB-DNA adducts were assessed over time and differences in individual adduct persistence were observed; the levels of O^2-POB-dThd increased over time, while the levels of 7-POB-Gua decreased over time (Lao *et al.*, 2007), suggesting that O^2-POB-

dThd is repaired relatively poorly *in vivo*. It would be of interest to determine if similar relative differences in adduct repair exist in human lung, as this will provide information as to which POB-DNA adducts may be more carcinogenic to humans based on their relative resistance to repair.

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