SOME TOBACCO-SPECIFIC *N*-NITROSAMINES

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Four tobacco-specific *N*-nitrosamines (TSNA), namely, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), *N*′-nitrosonornicotine (NNN), *N*′-nitrosoanabasine (NAB) and *N*′-nitrosoanatabine (NAT) were considered by a previous Working Group in October 1984 (IARC, 1985). Since that time, new data have become available and are presented in this monograph.

In addition to these compounds, new TSNA have been identified (Figure 1) and their concentrations in tobacco and tobacco smoke have been assessed. The occurrence of 4-(methylnitrosamino)-4-(3-pyridyl)butyric acid (*iso*-NNAC), 4-(methylnitrosamino)-4-(3-pyridyl)-1-butanol (*iso*-NNAL) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) in tobacco is reported in the monograph on Smokeless Tobacco; however, as a result of the limited data available, these TSNA have not been considered in the present evaluation.

This monograph does not consider the exposure of workers involved in the production of these compounds, which are used solely for laboratory research purposes.

1. Exposure Data

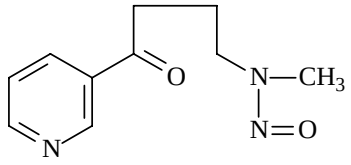
4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)

1.1 Chemical and physical data

1.1.1 *Synonyms and trade names*

Chem. Abstr. Services Reg. No.: 64091-91-4
Chem. Abstr. Name: 1-Butanone, 4-(methylnitrosoamino)-1-(3-pyridinyl)-
IUPAC Systematic Name: 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone
Synonym: 4-(*N*-Methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone

1.1.2 *Structural and molecular formulae and relative molecular mass*



$C_{10}H_{13}N_3O_2$ Relative molecular mass: 207.2

1.1.3 *Chemical and physical properties of the pure substance*

From Toronto Research Chemicals (2005, 2006), unless otherwise specified

(*a*) *Description:* Light-yellow crystalline solid

(*b*) *Melting-point:* 61–63 °C

(*c*) *Spectroscopy data*: Infrared, nuclear magnetic resonance and mass spectra have been reported (IARC, 1985).

(*d*) *Solubility*: Soluble in dichloromethane, dimethyl sulfoxide (DMSO), dimethylfuran, ethyl acetate and methanol

(*e*) *Stability*: Sensitive to light

N′-Nitrosonornicotine (NNN)

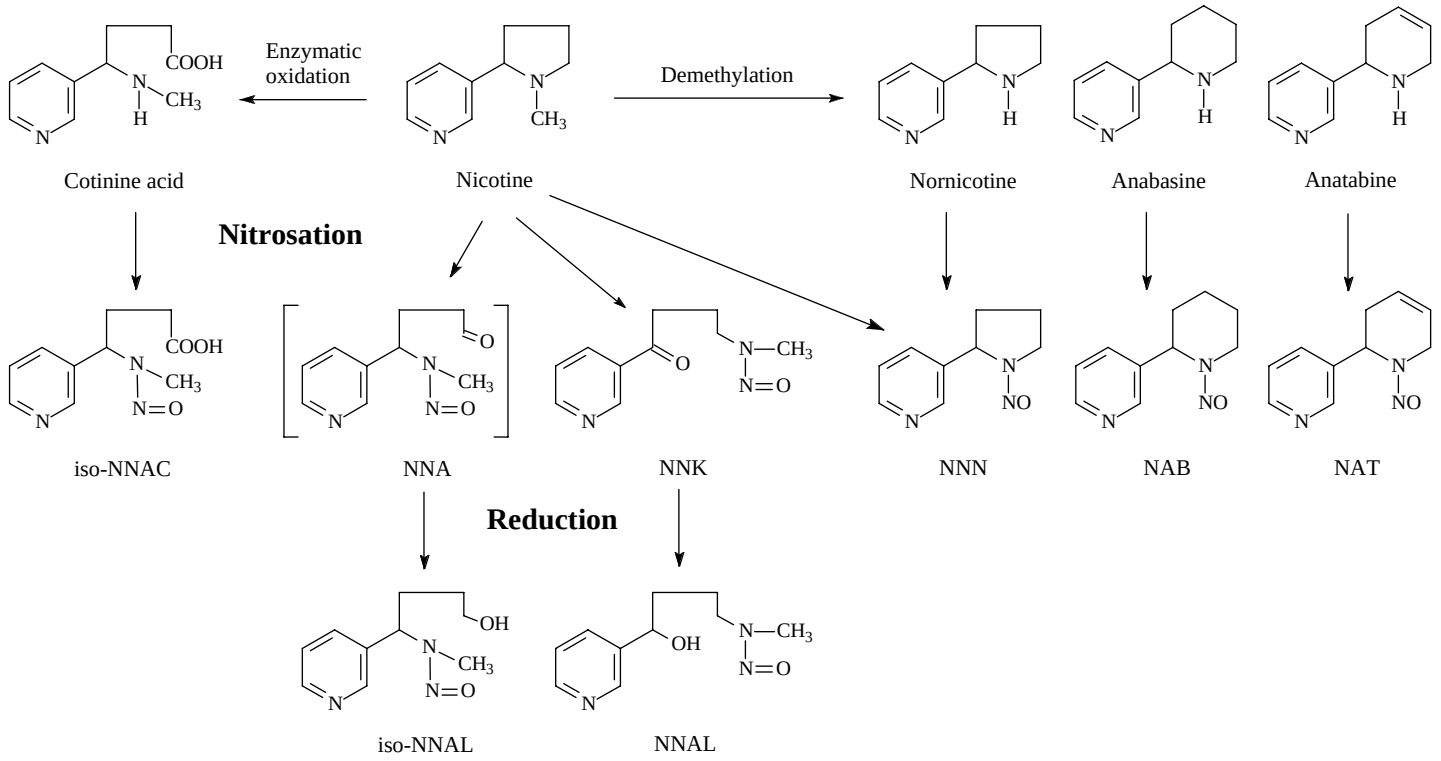
1.1 Chemical and physical data

1.1.1 *Synonyms and trade names*

Chem. Abstr. Services Reg. Nos: 80508-23-2; 16543-55-8[1]; 84237-38-7[2]
Chem. Abstr. Names: Pyridine, 3-(1-nitroso-2-pyrrolidinyl)-; pyridine, 3-(1-nitroso-2-pyrrolidinyl)-,(S)-[1]; pyridine, 3-(1-nitroso-2-pyrrolidinyl)-, (+,–)-[2]
IUPAC Systematic Name: 1′-Demethyl-1′-nitrosonicotine
Synonyms: 1′-Demethyl-1′-nitrosonicotine; 1′-desmethyl-1′-nitrosonicotine; 1′-nitroso-1′-demethylnicotine; nitrosonornicotine; *N*-nitrosonornicotine; 1′-nitrosonornicotine; 1-nitroso-2-(3-pyridyl)pyrrolidine; 3-(1-nitroso-2-pyrrolidinyl)pyridine

[1] The Chemical Abstracts Services Registry Number and Name refer to the (S) stereoisomer.
[2] The Chemical Abstracts Services Registry Number and Name refer to the racemic mixture that was synthesized and used in the biological studies reported in this monograph.

Figure 1. Formation of tobacco-specific *N*-nitrosamines

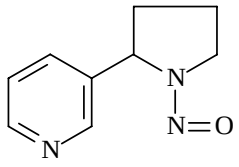


From Hoffmann *et al.* (1995)

iso-NNAC, 4-(methylnitrosamino)-4-(3-pyridyl)butyric acid; *iso*-NNAL, 4-(methylnitrosamino)-4-(3-pyridyl)-1-butanol; NAB, *N*′-nitrosoanabasine; NAT, *N*′-nitrosoanatabine; NNA, 4-(methylnitrosamino)-4-(3-pyridyl)butanal; NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNN, *N*′-nitrosonornicotine

Note: NNA is a very reactive aldehyde and has therefore never been quantified in tobacco or tobacco smoke.

1.1.2 *Structural and molecular formulae and relative molecular mass*



$C_9H_{11}N_3O$ Relative molecular mass: 177.2

1.1.3 *Chemical and physical properties of the pure substance*

From Toronto Research Chemicals (2005, 2006), unless otherwise specified

(*a*) *Description*: Light-yellow oil
(*b*) *Boiling-point*: 154 °C at 0.2 mm (IARC, 1985)
(*c*) *Melting-point*: 47 °C (IARC, 1985); 42–45 °C
(*d*) *Spectroscopy data*: Mass, ultraviolet, infrared and nuclear magnetic resonance spectra have been reported (IARC, 1985).
(*e*) *Solubility*: Soluble in acetone and chloroform
(*f*) *Stability*: Hygroscopic

N′-Nitrosoanabasine (NAB)

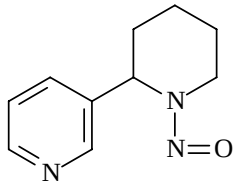
1.1 Chemical and physical data

1.1.1 *Synonyms and trade names*

Chem. Abstr. Services Reg. Nos: 37620-20-5; 1133-64-8[1]; 84237-39-8[2]
Chem. Abstr. Names: Pyridine, 3-(1-nitroso-2-piperidinyl)-; pyridine, 3-(1-nitroso-2-piperidinyl)-,(S)-[1]; pyridine, 3-(1-nitroso-2-piperidinyl), (+,–)-[2]
UPAC Systematic Name: 1-Nitrosoanabasine
Synonym: N-Nitrosoanabasine

[1] The Chemical Abstracts Services Registry Number and Name refer to the (S) stereoisomer.
[2] The Chemical Abstracts Services Registry Number and Name refer to the racemic mixture that was synthesized and used in the biological studies reported in this monograph.

1.1.2 *Structural and molecular formulae and relative molecular mass*



$C_{10}H_{13}N_3O$ Relative molecular mass: 191.2

1.1.3 *Chemical and physical properties of the pure substance*

From Toronto Research Chemicals (2005, 2006), unless otherwise specified

(*a*) *Description*: Yellow oil
(*b*) *Boiling-point*: 162 °C at 1 mm Hg (IARC, 1985); 165–167 °C at 0.25 mm Hg
(*c*) *Optical rotation*: The specific rotation of NAB has been reported (IARC, 1985)
(*d*) *Spectroscopy data*: Infrared, ultraviolet, nuclear magnetic resonance and mass spectra have been reported (IARC, 1985).
(*e*) *Solubility*: Soluble in chloroform and dichloromethane

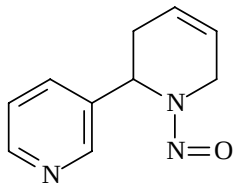
N′-Nitrosoanatabine (NAT)

1.1 Chemical and physical data

1.1.1 *Synonyms and trade names*

Chem. Abstr. Services Reg. No.: 71267-22-6
Chem. Abstr. Name: 2,3′-Bipyridine, 1,2,3,6-tetrahydro-1-nitroso-
IUPAC Systematic Name: 1,2,3,6-Tetrahydro-1-nitroso-2,3-bipyridine
Synonym: NAtB

1.1.2 *Structural and molecular formulae and relative molecular mass*



$C_{10}H_{11}N_3O$ Relative molecular mass: 189.2

1.1.3 *Chemical and physical properties of the pure substance*

From Toronto Research Chemicals (2005, 2006), unless otherwise specified

(*a*) *Description:* Pale-yellow oil
(*b*) *Boiling-point*: 176 °C at 0.5 mm Hg (IARC, 1985)
(*c*) *Spectroscopy data*: Infrared, mass and nuclear magnetic resonance spectra have been reported (IARC, 1985).
(*d*) *Solubility*: Soluble in chloroform, ethanol and methanol
(*e*) *Stability*: Sensitive to light

1.2 Technical products and impurities, analysis, production and use

1.2.1 *Technical products and impurities*

NNK, NNN, NAB and NAT are available commercially at a purity of 98%, in units of up to 1 g (Toronto Research Chemicals, 2006). NNK and NNN isolated from tobacco are mixtures of 72.7% E-isomer and 27.3% Z-isomer (Hecht *et al.*, 1977; Hoffmann *et al.*, 1980).

1.2.2 *Analysis*

Standard methods for the analysis of NNK, NNN, NAB and NAT have been described previously (Egan *et al*., 1983). Since that time, numerous studies on the levels of TSNA in tobacco and tobacco smoke have included descriptions of methods for the extraction and quantification of TSNA (Andersen *et al*., 1989; Djordjevic *et al*., 1989a; Fischer & Spiegelhalder, 1989; Fischer *et al*., 1989a; Spiegelhalder *et al*., 1989; Sharma *et al.*, 1991; Stepanov *et al*., 2002; Ashley *et al*., 2003; Jansson *et al.*, 2003; Wu *et al*., 2003, 2004; Stepanov *et al*., 2005; Wu *et al*., 2005; Stepanov *et al*., 2006a).

Risner *et al*. (2001) reported a collaborative investigation of methods for the determination of TSNA in tobacco. Seventeen laboratories around the world participated in this study and used seven different methods or variations thereof. The methods varied in sample preparation, conditions of analysis, compound detection and quantification of results. Morgan *et al*. (2004) reported the results from a collaborative study carried out by 15 different laboratories that used two different methods to determine TSNA. Both methods proved to be efficient for the determination of TSNA in a variety of tobacco types.

Methods for the assessment of NNAL, *iso*-NNAL and *iso*-NNAC in tobacco have also been described (Brunnemann *et al*., 1987a; Djordjevic *et al*., 1989a; Djordjevic *et al*., 1993a).

Methods for determination of TSNA in mainstream tobacco smoke are also available on various websites, such as those from Health Canada (http://www.hc-sc.gc.ca/hl-vs/tobac-tabac/legislation/reg/indust/method/index_e.html) or from the United Kingdom Government Benchmark Study (http://www.the-tma.org.uk/benchmark/).

1.2.3 *Production*

NNK was first prepared by the reaction of sodium hydroxide and sodium nitrite with 4-(*N*-methyl)-1-(3-pyridyl)-1-butanone dihydrochloride (IARC, 1985). NNK is currently produced commercially (Toronto Research Chemicals, 2005, 2006).

NNN was first prepared by treating nornicotine with sodium nitrite in dilute hydrochloric acid (IARC, 1985). NNN is currently produced commercially (Toronto Research Chemicals, 2005, 2006).

NAB was first prepared by treating anabasine with sodium nitrite in dilute hydrochloric acid solution (IARC, 1985). NAB is currently produced commercially (Toronto Research Chemicals, 2005, 2006).

NAT was first prepared by the reaction of sodium nitrite with a solution of anatabine in hydrochloric acid (IARC, 1985). NAT is currently produced commercially (Toronto Research Chemicals, 2005, 2006).

iso-NNAC was synthesized from cotinine *via* 4-(methylamino)-4-(3-pyridyl)butyric acid and *N*-nitrosated. Currently, *iso*-NNAC, *iso*-NNAL and NNAL are also produced commercially as well (Toronto Research Chemicals, 2005, 2006).

1.2.4 *Use*

No evidence was found that NNK, NNN, NAB or NAT have ever been used other than in laboratory research that included animal studies (Hoffmann *et al*., 1993a; Hecht, 1998).

1.3 Occurrence

The wide disparity in levels of TNSA in the mainstream smoke of cigarettes (Counts *et al*., 2005; Gregg *et al.*, 2005; King *et al.*, 2007) and in smokeless tobacco products (Hoffmann *et al*., 1995; Stepanov *et al*., 2006a) is largely due to differences in the tobacco types used in any given product, agricultural practices, curing methods and manufacturing processes (IARC, 2004). While trace amounts of TSNA were measured in green tobacco leaves (Bhide *et al*., 1987; Djordjevic *et al*., 1989b), these compounds are formed from their alkaloid precursors and from nitrite or nitrate predominantly during tobacco curing, fermentation and ageing (Burton *et al*., 1989a,b; Djordjevic *et al*., 1993a). The type of tobacco (e.g., Burley, Bright, Virginia or Oriental), its nitrate and nitrite content, the mode of curing (e.g. air-, flue- or sun-curing) and the various steps of processing used are therefore the determining factors for the yields of TSNA in tobacco (Fischer *et al.*, 1989a; Bush *et al*., 2001; Peele *et al*., 2001). NNN, NAB and NAT are formed primarily from their corresponding secondary amines (namely nornicotine, anatabine and anabasine) in the early stages of tobacco curing and processing whereas the majority of NNK and some NNN are formed from the tertiary amine nicotine at the later stages of tobacco curing and fermentation (Spiegelhalder & Fischer, 1991).

Levels of NNN and other TSNA are consistently higher in Burley than in Bright tobacco, regardless of the curing method (Chamberlain & Chortyk, 1992; Morgan *et al*., 2004). However, flue-curing of Bright tobacco produces nearly three times more TSNA than air-curing of the same tobacco. TSNA content varies between the parts of the plant. For example, NNK and NNN contents are higher in the midrib than in the lamina in air-cured tobacco, whereas the converse is observed in flue-cured tobacco. Flue-curing of Burley tobacco reduces the alkaloid content but greatly increases TSNA content in the lamina. Midribs from air-cured Burley leaves contain three times the TSNA concentrations of the lamina (Chamberlain & Chortyk, 1992). Analyses of 41 leaf segments from a dark air-cured tobacco variety (KY 171) revealed that concentrations of the individual nitrosamines were lowest at the tip and the periphery of the leaf (Burton *et al*., 1992). The midvein contained the lowest concentrations of NNK, NNN and NAT at two-thirds of the length of the leaf; the highest concentrations were found at the base of the leaf. The correlation between nitrite nitrogen and TSNA was higher than that between alkaloid content and TSNA in the leaf.

DeRoton *et al*. (2005) summarized the major factors involved in the formation of TSNA in dark air-cured and Burley tobacco during curing and post-curing treatment. The main genetic trait involved in the formation of TSNA is the propensity of a variety of tobacco to convert nicotine to nornicotine. In addition, the ability of a variety to lose water rapidly limits the formation of nitrite and hence also the formation of TSNA. Since TSNA are derived from tobacco alkaloids, agricultural practices that increase alkaloid concentrations in the tobacco leaves favour their formation. Air flow at the site of curing also influences the levels of TSNA: leaves that are cured in well-ventilated curing structures, such as plastic sheds, generally contain lower amounts of TSNA than those cured in barns. Levels of TSNA may increase after curing if tobacco leaves are stored under humid conditions (Staaf *et al*., 2005) or in bales. The biological mechanism for TSNA formation in air-cured tobacco relates to the breakdown of plant cell membranes due to moisture loss, which makes the cell contents available to microorganisms that produce nitrite. That is, microbes generate nitrite as a by-product, and this becomes available to react with alkaloids to form TSNA. When curing is begun with relatively high humidity at the yellowing phase followed by a pronounced decline as cell breakdown begins, the TSNA content in tobacco will be lower. A uniform air-flow increases the rate and amount of moisture loss from the tobacco, and also reduces possible gas-phase reactions between alkaloids and gaseous nitric oxides.

Microorganisms appear to play a lesser role in TSNA formation in flue-cured tobacco. Morin *et al*. (2004) found that microbial populations were inversely correlated with concentrations of TSNA and with temperature. However, TSNA concentrations increased as temperature increased during curing. This finding supports the hypothesis that TSNA could result from the reaction of combustion gases (e.g. nitrogen oxides) with tobacco alkaloids during flue-curing. Removal of heating with propane as part of the curing process has been shown to reduce the levels of NNK and NNN substantially (Peele *et al*., 2001; IARC, 2004).

In summary, a lesser degree of fertilization, particularly with products that contain nitrates, and careful manipulation of curing parameters and tobacco blending can lower the level of nitrosamines in tobacco products.

1.3.1 *Fresh tobacco*

The mean concentrations of NNK, NNN and NAT in the green leaves harvested at all stalk positions from the flue-cured tobacco plant NC-95 were 280, 260 and 790 ng/g dry tobacco, respectively (Djordjevic *et al.*, 1989b), and were six times higher in cured tobacco (namely 1810, 1560 and 6670 ng/g dry tobacco, respectively).

Bhide *et al.* (1987) reported the presence of NNK and NNN in green leaves of *N. tabacum* and *N. rustica* species grown in India in two different seasons. In one season, mature green leaves of *N. rustica* contained up to 2340, 46 100, 5200 and 23 700 ng/g tobacco (dry wt) NNK, NNN, NAB and NAT, respectively. One year later, tobacco harvested at the same location contained 352 ng/g tobacco NNK and 5730 ng/g tobacco NNN. These levels rose to 25 800 ng/g tobacco NNK and 15 000 ng/g tobacco NNN in sun-dried tobacco. In comparison, the levels of TSNA in sun-dried *N. tabacum* species grown in the same area during the same seasons were 37 ng/g tobacco NNK and 49 ng/g tobacco NNN.

1.3.2 *Cured tobacco*

A wide range of TSNA concentrations are found in cured tobacco, regardless of the type (Table 1). In each category of tobacco type, the range reflects the diversity of the tobacco variety, production year, climate, country of origin, agricultural practices including fertilization, post-harvesting and curing technologies, post-curing handling and storage conditions, as well as the analytical methods used and the reporting of the analytical results (e.g. ng/g dry tobacco wt versus ng/g wet tobacco wt).

The levels of total TSNA are highest in air-cured Burley tobacco and lowest in sun-cured Oriental (Turkish) tobacco. The highest levels of NNN were reported in Burley laminae and midribs (up to 8620 and 9080 ng/g dry tobacco, respectively). The highest reported concentrations of NNN were 1700 ng/g dry wt in flue-cured Bright tobacco and 420 ng/g dry wt in sun-cured Oriental tobacco. MacKown *et al.* (1988) also reported levels of NNN up to 3400 ng/g dry wt in reconstituted tobacco sheets that are used in cigarette blends.

The highest levels of NNK were reported in midribs of Burley tobacco (6660 ng/g dry tobacco) and laminae of Bright (Virginia) tobacco (2690 ng/g dry tobacco). It should be noted that levels of NNK in Burley midribs exceed those in the laminae (6600 versus 1370 ng/g dry wt). It should also be noted that NNK is a predominant TSNA in Bright tobacco (2690 ng NNK compared with 1370 ng NNN) while NNN is predominant in Burley tobacco (1370 ng NNK compared with 8620 ng NNN). Similarly to NNN, NAB and NAT are formed by nitrosation of a respective secondary amine alkaloid precursor

Table 1. The concentration ranges of tobacco-specific *N*-nitrosamines in cured tobacco produced worldwide

Tobacco type	Reported as[a]	NNK	NNN	NAB	NAT	NAB + NAT	Reference
Oriental (Turkish)	Dry	ND–83[b]	20–420	50	20–170		Djordjevic *et al.* (1991); Morgan *et al.* (2004)
	Wet	ND–70[c]	20–460			20–200	Fischer *et al.* (1989a)
Bright (Virginia)	Dry	160–2690	240–1700	20–150	280–6670		Djordjevic *et al.* (1989b); Risner *et al.* (2001); Morgan *et al.* (2004)
	Wet	30–1100	10–600			30–950	Fischer *et al.* (1989a)
Burley (laminae)	Dry	ND–1370	1070–8620	200–223	4270–19 700		MacKown *et al.* (1988); Burton *et al.* (1989a); Risner *et al.* (2001); Morgan *et al.* (2004)
	Wet	100–1400	1300–8850			500–3600	Fischer *et al.* (1989a)
Burley (stems/midrib)	Dry	ND–6660	1500–9080	155	1800–20 940		MacKown *et al.* (1988); Burton *et al.* (1989a); Morgan *et al.* (2004)
Reconstituted tobacco sheets	Dry	490	3400		2500		MacKown *et al.* (1988)

NAB, *N*′-nitrosoanabasine; NAT, *N*′-nitrosoanatabine; ND, not detected; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNN, *N*′-nitrosonornicotine

[a] Dry, ng/g dry wt; wet, ng/g wet wt

[b] Detection limit for NNK, < 10 ng/g tobacco

[c] Detection limit for NNK, < 50 ng/g tobacco

(Figure 1), and their accumulation in tobacco follows that of NNN: the highest concentrations were reported in Burley tobacco and the lowest in Oriental tobacco.

Some authors did not report concentrations of NAB and NAT separately but as a sum (Fischer *et al*., 1989a). In some reports, TSNA values are expressed per dry tobacco wt while values are not adjusted for the moisture content in others. The variability of the data thus reflects not only diversity of tobacco specimens but also of analytical methods and instrumentation. There is an urgent need for standardization and validation of analytical methods for measurements of TSNA in tobacco, including reporting of results, to permit a meaningful comparison of data.

1.3.3 *Cigarette tobacco*

Table 2 presents a comparison of concentrations of NNK, NNN, NAB and NAT in tobacco from commercial cigarettes sold worldwide. NNK ranged from not detected to 10 745 ng/cigarette in cigarettes sold in Italy (Fischer *et al*., 1990a). NNK was not detected in some cigarettes sold in the Central Europe, Middle East and Africa region, the European Union and the former USSR (Fischer *et al.*, 1990a; Djordjevic *et al.*, 1991; Counts *et al*., 2005). High concentrations of NNK were also reported for cigarettes sold in India (4800 ng/g dry tobacco), the USA (1760 ng/g wet tobacco) and France (1530 ng/g dry tobacco).

NNN ranged from 20 ng/g dry tobacco for cigarettes sold in the former USSR (Djordjevic *et al*., 1991) to 58 000 ng/g dry tobacco for cigarettes from India (Nair *et al*., 1989). High concentrations of NNN were also reported in cigarettes sold in France (18 600 ng/g dry tobacco), the USA (up to 7900 ng/g dry tobacco), Germany (up to 5340 ng/cigarette), Poland (up to 4870 ng/g dry tobacco), Japan (up to 3892 ng/g dry tobacco), Norway (3736 ng/g dry tobacco) and Malaysia (up to 3350 ng/g dry tobacco).

NAB ranged from not detectable levels to 322 ng/g dry tobacco. The highest amount was measured in Philip Morris cigarettes marketed in the Central Europe, Middle East and Africa region (Counts *et al*., 2005).

NAT ranged from 20 to 15 100 ng/g dry tobacco. The lowest concentrations were reported for cigarettes from the former USSR (Fisher *et al*., 1990a; Djordjevic *et al*., 1991) and the highest concentration for an Indian cigarette (Nair *et al*., 1989). High levels of NAT were also reported in cigarettes from France, the Central Europe, Middle East and Africa region, the European Union, Japan, Taiwan (China) and the USA.

Ashley *et al*. (2003) measured TSNA in tobacco from cigarettes purchased in 21 countries. US brands of cigarettes marketed worldwide generally had higher levels than popular local cigarettes in many countries.

Higher TSNA concentrations were generally measured in the tobacco from non-filter cigarettes, especially those made of dark tobacco (Fischer *et al*., 1989a; Tricker *et al*., 1991). Among the 55 brands sold in Germany in 1987 (Table 3), the lowest amounts of NNK and NNN were measured in cigarettes made from Oriental tobacco (NNK, not detectable to 177 ng/cigarette; NNN, 45–432 ng/cigarette), followed by cigarettes made with Virginia tobacco (NNK, 170–580 ng/cigarette; NNN, 133–330 ng/cigarette) and

Table 2. International comparison of the concentration ranges for preformed tobacco-specific *N*-nitrosamines in tobacco from commercial cigarettes

Country	Reported as[a]	NNK	NNN	NAB	NAT	NAB + NAT	Reference
Argentina	Dry	812	1866	357	1559		Counts *et al.* (2005)
Australia	Dry	490–1193	420–2888	NQ[b]–207	715–2366		Counts *et al.* (2005)
Austria	ng/cig	92–310	306–1122				Fischer *et al.* (1990a)
Belgium	ng/cig	219–594	504–1939				Fischer *et al.* (1990a)
Canada	ng/cig	447–884	259–982			564–1017	Fischer *et al.* (1990b)
CEMA	Dry	NQ[b]–1127	1094–3739	NQ[b]–322	1014–2989		Counts *et al.* (2005)
European Union	Dry	NQ[b]–860	332–2736	NQ[b]–262	423–2253		Counts *et al.* (2005)
France	Dry ng/cig	260–1530 57–990	4770–18 600 120–6019	100	1200–9970		Djordjevic *et al.* (1989a); Ohshima *et al.* (1985); Fischer *et al.* (1990a)
Germany	ng/cig Dry	ND–1120[c] 445–469	45–5340 1355–1361	 NQ[b]	 1142–1207	ND–2490[c]	Fischer *et al.* (1989a, 1990a); Tricker *et al.* (1991); Counts *et al.* (2005)
India	Dry ng/cig	40–4800 19–174	1300–58 000 68–730		800–15 100	 98–519[c]	Nair *et al.* (1989); Kumar *et al.* (1991)
Italy	ng/cig	153–10 745	632–12 454				Fischer *et al.* (1990a)
Japan	Dry	190–1171	360–3892	NQ[b]–320	300–3139		Djordjevic *et al.* (2000a); Counts *et al.* (2005)
Malaysia	Dry	434–923	2223–3350	NQ[b]–176	1302–2170		Counts *et al.* (2005)
Moldova	Wet	104–942	93–2090	ND–75	55–1290		Stepanov *et al.* (2002)
Netherlands	ng/cig	105–587	58–1647				Fischer *et al.* (1990a)
Norway	Dry	1124	3736	379	2945		Counts *et al.* (2005)

Table 2 (contd)

Country	Reported as[a]	NNK	NNN	NAB	NAT	NAB + NAT	Reference
Poland	Dry ng/cig	70–660 140–450	670–4870 870–2760				Fischer *et al.* (1990a); Djordjevic *et al.* (2000b)
Sweden	ng/cig	192–569	544–1511				Fischer *et al.* (1990a)
Switzerland	ng/cig	450–554	1280–2208				Fischer *et al.* (1990a)
Taiwan	Dry	1194	3769	195	2883		Counts *et al.* (2005)
United Kingdom	ng/cig	92–433	140–1218				Fischer *et al.* (1990a)
USA	Dry Wet ng/cig	420–1270 1410–1760 433–733	880–7900 2590–4300 993–1947	ND–212 100–140	880–5810 1610–2660		Ohshima *et al.* (1985); Djordjevic *et al.* (1990); Fischer *et al.* (1990a); Djordjevic *et al.* (2000a); Stepanov *et al.* (2002); Counts *et al.* (2005)
Former USSR	Dry ng/cig	ND–40[d] ND–150[c]	20–420 60–850		20–170		Fischer *et al.* (1990a); Djordjevic *et al.* (1991)
Venezuela	Dry	591	2170	244	1339		Counts *et al.* (2005)

Adapted from IARC (2004)

CEMA, Central Europe, Middle East, Africa; NAB, *N*′-nitrosoanabasine; NAT, *N*′-nitrosoanatabine; ND, not detected; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNN, *N*′-nitrosonornicotine; NQ, not quantifiable

[a] Dry, ng/g dry tobacco; ng/cig, ng/cigarette; wet, ng/g wet tobacco

[b] Limit for quantitation; NNK, 272 ng/g; NNN, 180 ng/g; NAB, 103 ng/g; NAT, 213 ng/g

[c] Detection limit for NNK, < 50 ng/cigarette; for NNN and for NAB + NAT < 25 ng/cigarette

[d] Detection limit for NNK, < 10 ng/g tobacco

Table 3. International comparison of the concentration ranges of preformed tobacco-specific *N*-nitrosamines in tobacco from commercial cigarettes with a wide range of ISO/FTC nicotine and 'tar' mainstream smoke yields[a]

Country (total no. of cigarette brands in the study)	Reported as[b]	Tobacco filler	F/NF	NNK	NNN	NAB	NAT	NAB + NAT	Reference
Canada	ng/cig	Ultra-low yield (V)	F	447–785	288–982			666–1017	Fischer *et al.* (1990b)
(25)		Low yield (V)	F	510–884	292–527			586–978	
		Moderate yield (V)	F	569–705	337–407			666–779	
		High yield (V)	F	495–663	259–381			564–758	
Germany	ng/cig	Blend	F	100–410	400–1390			220–1340	Tricker *et al.* (1991)
(20)		Blend	NF	270–500	660–2670			460–1110	
		Dark	NF	800–960	4500–5340			1650–2330	
(55)		Oriental	F + NF	ND–177	45–432			ND–575	Fischer *et al.* (1989a,b)
		Virginia	F + NF	170–580	133–330			253–630	
		American blend	F	160–696	500–2534			440–2490	
		Dark	NF	370–1120	3660–5316			266–315	
Japan	Dry	Low yield	F	190–330	810–1110	30–60	410–660		Djordjevic *et al.* (2000a)
(6)		Medium yield	F	200–320	360–1040	30–70	300–620		
USA	Dry	Ultra-low yield (AB)	F	500–580	1750–1980	ND	970–1080		Djordjevic *et al.* (1990, 2000a)
(13)		Low yield (AB)	F	490–800	1900–3050	90–120	1030–1670		
		Moderate yield (AB)	F	420–890	1780–2890	70–110	1030–1680		
		High yield (AB)	NF	770–920	1290–2160	40–110	920–1170		
(11)	Wet	Quest 1,2,3[c]	F	54–190	820–930	3–13	43–310		Stepanov *et al.* (2006a)
		Ultra-low yield (AB)	F	750–770	2800–2900	55–58	1100–1200		
		Low yield (AB)	F	550–680	2700–2800	51–61	1100–1300		
		Moderate yield (AB)	F	580–960	1100–2900	25–100	560–2300		

Table 3 (contd)

Country (total no. of cigarette brands in the study)	Reported as[b]	Tobacco filler	F/NF	NNK	NNN	NAB	NAT	NAB + NAT	Reference
Philip Morris commercial brands (39)	Dry	Ultra-low yield (AB)	F	NQ[d]–1171	420–3892	NQ[d]–262	715–3139		Counts *et al.* (2005)
		Low yield (AB)	F	NQ[d]–1270	332–3438	NQ[d]–322	423–2676		
		Moderate yield (AB)	F	NQ[d]–1194	868–3769	NQ[d]–379	778–2945		

AB, American blend cigarettes; F, filter-tipped cigarettes; NAB, *N*′-nitrosoanabasine; NAT, *N*′-nitrosoanatabine; ND, not detected; NF, non-filtered cigarettes; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNN, *N*′-nitrosonornicotine; NQ, not quantifiable; V, Virginia-type cigarettes

[a] Cigarettes were designated into classes based either on ISO/FTC nicotine smoke yields (Djordjevic *et al.*, 2000a) or ISO/FTC 'tar' smoke yields (IARC, 1986).

[b] Dry, ng/g dry tobacco; ng/cig, ng/cigarette; wet, ng/g wet tobacco

[c] Nicotine-reduced cigarettes

[d] Limit for quantitation: NNK, 272 ng/g; NAB, 103 ng/g

American blend cigarettes (NNK, 160–696 ng/cigarette; NNN, 500–2534 ng/cigarette). The highest TSNA levels were reported in the dark tobacco cigarettes (NNK, 370–1120 ng/cigarette; NNN, 3660–5316 ng/cigarette) (Fischer *et al*., 1989a,b; Tricker *et al*., 1991).

Tobacco from ultra-low-, low-, medium- and high-yield cigarettes sold internationally, as ranked based on data obtained by the ISO/FTC (International Standardization Organization/Federal Trade Commission) machine-smoking method (IARC, 2004), contain similar amounts of preformed NNK and NNN regardless of the type of product (Table 3; Djordjevic *et al*., 1990; Fischer *et al.*, 1990b; Djordjevic *et al*., 2000a; Counts *et al*., 2005; Stepanov *et al*., 2006a). For example, levels of NNK and NNN in the tobacco of Canadian ultra-low-yield cigarettes ranged from 447 to 785 ng/cigarette and from 288 to 982 ng/cigarette, respectively, whereas high-yield cigarettes contained 495–663 ng/cigarette NNK and 259–381 ng/cigarette NNN (Fischer *et al*., 1990b). Similar observation was reported for American blend cigarettes sampled in the USA (Djordjevic *et al*., 1990, 2000a; Stepanov *et al*., 2006a) or internationally (Counts *et al*., 2005). Tobacco from Canadian brands with a wide range of nicotine and tar yields as measured by the ISO/FTC method contained lower amounts of preformed NNN (up to 982 ng/cigarette; Fischer *et al*., 1990b) than US brands sold domestically or internationally (up to 3050 and 3892 ng/g dry tobacco, respectively; Djordjevic *et al*., 1990, 2000a; Counts *et al*., 2005). In contrast, NNK content in cigarette tobacco was of the same order of magnitude in the two countries (up to 884 ng/cigarette in Canadian cigarettes and up to 920 ng/cigarette in cigarettes sold in the USA). However, Philip Morris American blend cigarettes marketed worldwide contained the highest quantities of both NNK and NNN (up to 1270 and 3892 ng/g dry tobacco, respectively). Japanese and German cigarettes contained the lowest concentrations of preformed NNK (up to 330 ng/g dry tobacco).

Typically, levels of NNK in a given cigarette blend are lower than those of NNN except in cigarettes manufactured in Canada, the United Kingdom and Australia that use Bright Virginia flue-cured tobacco as a filler; in the latter brands, NNK is the dominant TSNA (Fischer *et al*., 1989a,b, 1990b).

Cigarettes manufactured and marketed globally contain a wide range of TSNA. The dose of TSNA delivered in mainstream smoke is largely determined by their levels in the tobacco blend (Fischer *et al*., 1990c; d'Andres *et al*., 2003). In addition, exposure to TSNA depends on the relationship between the product and how it is used, that is, on smoking patterns and intensity (Djordjevic *et al*., 2000c; Hecht *et al.*, 2005; Melikian *at al*., 2007a,b).

1.3.4 *Mainstream cigarette smoke*

Monitoring of tobacco smoke has historically been limited to the measurement of carbon monoxide emissions from cigarettes using the ISO/FTC machine-smoking protocol, which does not reflect the characteristics of human smoking. During the past two decades, many studies have reported the occurence of TSNA in mainstream smoke. Table 4

Table 4. International comparison of the concentration ranges (ng/cigarette) of tobacco-specific *N*-nitrosamines in the mainstream smoke of commercial cigarettes (ISO/FTC machine-smoking method)

Country	NNK	NNN	NAB	NAT	NAB + NAT	Reference
Argentina	79.5	99.6	14.5	106.9		Counts *et al.* (2005)
Australia	12.4–106.1 25.7[a,b]	5.0–151.8 22.4[a,b]	2.0–22.3	8.0–134.7		Counts *et al.* (2005) King *et al.* (2007)
Austria	12–100	42–172				Fischer *et al.* (1990a)
Belgium	29–150	38–203				Fischer *et al.* (1990a)
Canada	6–97 50.9[a,c]	4–37 25.6[a,c]			9–82	Fischer *et al.* (1990b) King *et al.* (2007)
CEMA	18.0–75.0	42.5–147.5	6.0–18.5	38.6–129.2		Counts *et al.* (2005)
European Union	12.7–78.4	14.8–126.0	2.0–17.9	18.0–106.8		Counts *et al.* (2005)
Germany	ND[d]–470	5–855	6.6–9.0	39.9–52.6	6.6–520	Fischer *et al.* (1989b, 1990a); Tricker *et al.* (1991); Counts *et al.* (2005)
France	18.3–498	11–1000	15.4–18.7	127–182		Djordjevic *et al.* (1989a); Fischer *et al.* (1990a)
India	TR–73	6–401		3.8–99.4	18–146	Nair *et al.* (1989); Kumar *et al.* (1991)
Italy	8–1749	21–1353				Fischer *et al.* (1990a)
Japan	18.6–87.1	36–146	6.8–21	40.3–138		Djordjevic *et al.* (1996); Counts *et al.* (2005)
Malaysia	45.0–87.3	63.4–195.3	7.8–28.5	55.7–153.3		Counts *et al.* (2005)
Netherlands	5–102	9–163				Fischer *et al.* (1990a)
Norway	103.9	189.4	18.6	160.4		Counts *et al.* (2005)
Poland	36–990	68–2830				Fischer *et al.* (1990a); Gray *et al.* (1998); Djordjevic *et al.* (2000b)

Table 4 (contd)

Country	NNK	NNN	NAB	NAT	NAB + NAT	Reference
Sweden	27–84	44–141				Fischer *et al.* (1990a)
Switzerland	69–124	121–226				Fischer *et al.* (1990a)
Taiwan (China)	96.2	161.2	24.7	142.8		Counts *et al.* (2005)
Thailand	16–369	28–730			43.5–483	Brunnemann *et al.* (1996)
United Kingdom	5.2–500	6.5–257.6	1.1–44.2	7.8–148		Fischer *et al.* (1990a); Gregg *et al*. (2005)
USA	3–425	6–1007	ND–34.7	6–250	102–744	Adams *et al.* (1987a); Fischer *et al.* (1990a); Djordjevic *et al*. (1990); Brunnemann *et al.* (1994); Djordjevic *et al*. (1996)
Former USSR	4–55	23–389		71–196		Fischer *et al.* (1990a); Djordjevic *et al.* (1991)
Venezuela	64.2	100.1	13.3	78.6		Counts *et al.* (2005)

Adapted from IARC (2004)

CEMA, Central Europe, Middle East, Africa; NAB, *N*′-nitrosoanabasine; NAT, *N*′-nitrosoanatabine; ND, not detected; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNN, *N*′-nitrosonornicotine; TR, trace amounts

[a] Reported as ng/mg tobacco

[b] Mean of 15 brands

[c] Mean of 21 brands

[d] Limit of detection for NNK, 40 ng/cigarette

presents an international comparison of TSNA concentration ranges in mainstream smoke of commercial cigarettes using the ISO/FTC machine-smoking method.

Similarly to cigarette tobacco filler, the levels of individual TSNA in mainstream smoke vary dramatically among products regardless of the country of origin. The emissions of NNK ranged from undetected to 1749 ng/cigarette (Italy; Fischer *et al*., 1990a). The concentrations of NNN ranged from 4 ng/cigarette (Canada) to 2830 ng/cigarette (Poland; Gray *et al*., 1998). The highest emissions of NNN were reported in the mainstream smoke of cigarettes sold in Germany (855 ng/cigarette), France (1000 ng/cigarette), the USA (1007 ng/cigarette), Italy (1353 ng/cigarette) and Poland (2830 ng/cigarette). The concentrations of NAB ranged from undetected to 44.2 ng/cigarette and those of NAT from 6 to 250 ng/cigarette.

The lowest emissions of TSNA were measured in the mainstream smoke from blended cigarettes sold in Australia, Austria, Canada, Japan, the Netherlands and Sweden, with upper values of 66–106 ng NNK. Surprisingly, levels of NNK and NNN in the mainstream smoke of two cigarette brands from India were very low despite the extremely high levels of preformed nitrosamines in tobacco (Nair *et al*., 1989).

A comparative assessment of the composition of mainstream smoke from three popular brands of filter-tipped cigarette from the USA that were purchased on the open market in 29 countries worldwide showed from three- to ninefold differences in the yields of NNK and NNN within each brand. Yields of NNK and NNN were highly correlated (r = 0.88; Gray *et al*., 2000).

The parameters that affect smoke yields and compositions have recently been reviewed (Hoffman *et al*., 2001; Borgerding & Klus, 2005). Measurements of smoke yields using the ISO/FTC machine-smoking method do not provide information that is representative of the exposure of a smoker. Machine-smoking protocols other than the ISO/ FTC protocol have been examined, particularly those that have more intense puffing parameters which block some or all of the ventilation holes in cigarette filters. Examples include those developed by the Massachusetts Department of Public Health (MDPH) and by Health Canada (IARC, 2004). Table 5 depicts the differences in TSNA emissions in the mainstream smoke generated by machine smoking using the ISO/FTC, MDPH and Health Canada protocols. Thirty-nine Phillip Morris cigarettes marketed globally (Counts *et al*., 2005) were grouped into three categories (moderate-yield, low-yield and very low-yield) based on the ISO/FTC emissions of tar, nicotine and carbon monoxide (IARC, 1986, 2004). The range of levels of NNK, NNN, NAB, NAT and total TSNA in the emissions of each product category was wide regardless of the machine-smoking protocol. The levels of emissions within each protocol increased from very low-yield cigarettes to moderate-yield cigarettes. Since the tobaccos in each product category contain similar amounts of preformed TSNA per gram of tobacco (Table 3), the differences in emission levels are due solely to the characteristics of the cigarette design such as the amount of tobacco, length and circumference of a cigarette, perforation of the cigarette and filter paper, and the type of material used for filtration (Hoffmann & Hoffmann, 2001; Hoffmann *et al*., 2001; IARC, 2004; Borgerding & Klus, 2005; Counts *et al.*, 2005). It was calculated that, compared with the ISO/FTC conditions,

the MDPH method produces up to 2.2-fold higher yields of total TSNA for moderate-yield cigarettes, 2.7-fold higher yields for low-yield brands and threefold higher yields for ultra-low-yield brands. The ratios for the emissions using the Health Canada protocol follow the same trend but are somewhat higher (up to 2.4-, 3.4- and 5.2-fold, respectively) because of complete blockage of filter ventilation holes. Overall, MDPH and Health Canada protocols generally produce higher yields per cigarette and reduce the differences between brands in the yields.

Table 5. Comparison of the concentration ranges[a] of tobacco-specific *N*-nitrosamines (TSNA) in the mainstream smoke of 39 Philip Morris commercial brands marketed globally

Measurement conditions[b]	Cigarette type[c]		
	Moderate-yield	Low-yield	Very low-yield
ISO-condition			
NNK (ng/cig)	27.7–107.8	12.7–75	18–53.9
NNN (ng/cig)	48.1–195.3	14.8–147.5	5–103.5
NAB (ng/cig)	6–28.5	2–18.5	2–16.3
NAT (ng/cig)	44.1–160.4	18–129.2	8–91.2
Total TSNA (ng/cig)	127.1–472.3	47.5–370.2	27.4–264.9
MDPH-condition			
NNK (ng/cig)	60.6–208.6	36.2–150.4	25.8–130.8
NNN (ng/cig)	100.4–374.2	26.5–262.4	16.3–219.7
NAB (ng/cig)	10.5–46.9	4–27.7	5.8–33
NAT (ng/cig)	91.8–295.3	37.5–204.7	31.9–196.3
Total TSNA (ng/cig)	273.5–892.1	104.2–643.4	88.6–571.1
Health Canada-condition			
NNK (ng/cig)	73–263	44.7–171.1	39.1–157.5
NNN (ng/cig)	114.5–410.6	30.6–359.1	20.6–277.8
NAB (ng/cig)	13.8–50.1	5.3–42.8	4.3–33.1
NAT (ng/cig)	105.3–345.1	43.5–283.9	49.3–240.3
Total TSNA (ng/cig)	312.4–1049.8	124.1–852.8	113.3–708.7
Total TSNA ratios			
MDPH/ISO	1.7–2.2	1.7–2.7	2.1–3.0
Health Canada/ISO	1.9–2.5	1.9–3.4	2.7–5.2

From Counts *et al.* (2005)

NAB, *N*′-nitrosoanabasine; NAT, *N*′-nitrosoanatabine; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNN, *N*′-nitrosonornicotine

[a] The measurements are the averages of seven replicates within each machine-smoking method.

[b] Health Canada machine-smoking method; ISO, International Standardization Organization machine-smoking method; MDPH, Massachusetts Department of Public Health machine-smoking method

[c] Cigarettes were designated into classes based on ISO/FTC 'tar' smoke yields (IARC, 1986).

Table 6 presents an overview of the range of TSNA concentrations in mainstream and sidestream smoke of 26 major US cigarette brands as determined using the MDPH machine-smoking protocol. The concentrations of each individual TSNA vary substantially between cigarette brands marketed in the USA; these values are comparable to those reported for Philip Morris American blended cigarettes marketed globally (Table 5; Counts *et al*., 2005).

Table 6. Range of concentration of tobacco-specific *N*-nitrosamines in the mainstream and sidestream smoke of 26 US commercial cigarette brands (MDPH machine-smoking method)

	Concentration (ng/cigarette)		
	Mainstream	Sidestream	Sidestream/mainstream ratio
NNK	53.5–220.7	50.7–96.7	0.40
NNN	99.9–317.3	69.8–115.2	0.43
NAB	14.2–45.3	11.9–17.8	0.55
NAT	95.2–298.6	38.4–73.4	0.26

From Borgerding *et al.* (2000)
MDPH, Massachusetts Department of Public Health; NAB, *N*′-nitrosoanabasine; NAT, *N*′-nitrosoanatabine; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNN, *N*′-nitrosonornicotine

One study assessed the concentration of NNK in mainstream smoke using the smoking pattern of smokers. Among 133 smokers of low- or medium-yield cigarettes (Djordjevic *et al*., 2000c), smokers of low-yield brands drew somewhat larger puffs (48.6 versus 44.1 mL) and more smoke both per cigarette (615 mL versus 523 mL) and daily (9.5 L versus 8.2 L) compared with smokers of medium-yield cigarettes; however, concentrations of NNK in mainstream smoke were marginally higher among smokers of medium-yield cigarettes compared with smokers of low-yield brands (250.9 versus 186.5 ng/cigarette).

The most recent report examined whether differences in gender and ethnicity exist in relation to exposure to selected mainstream cigarette smoke constituents as a result of variations in smoking behaviour or type of cigarettes smoked among 129 female and 128 male smokers (Melikian *et al*., 2007a). Compared with men, women took smaller puffs (37.6 versus 45.8-mL puff; $p = 0.0001$) of shorter duration (1.33 versus 1.48-s puff; $p = 0.002$) but drew more puffs from their cigarettes (13.5 versus 12.0; $p = 0.001$) and smoked less of their cigarette (left 36.3-mm butts or 40.2% of cigarette length versus 34.3-mm butts or 39.2% of cigarette length; $p = 0.01$). The daily dose of smoke was significantly higher in men (9.3 versus 8.0 L; $p = 0.02$). When data were stratified by ethnicity, no difference was found in puffing characteristics between Caucasian American and African–American smokers, except that women and men in the latter group smoked their cigarettes

to an equal length (butt lengths, 34.5 versus 33.9 mm; $p = 0.93$). However, because African–Americans smoked fewer cigarettes, the daily smoke volume was significantly higher among Caucasian American smokers (8.61 versus 7.45 L for women; 10.6 versus 7.8 L for men). The emissions of selected compounds per cigarette, as determined by mimicking human smoking behaviours, were greater among the male smokers than among the female smokers and correlated significantly with delivered smoke volume per cigarette. Cigarettes smoked by women yielded 139.5 ng/cigarette NNK compared with 170.3 ng for men ($p = 0.0007$). The gender differences with regard to cigarette smoke delivery were more profound in Caucasian Americans than in African–Americans. On average, the smoking behaviour of African–American men produced the highest emissions and that of Caucasian American women produced the lowest.

1.3.5 *Sidestream cigarette smoke*

Adams *et al*. (1987a) determined the levels of TNSA in the mainstream and sidestream smoke of four different types of US commercial cigarette brands (untipped and filter-tipped, with or without filter perforation) with a wide range of ISO/FTC yields. The highest levels of NNK and NNN were measured in the sidestream smoke of untipped cigarettes (1444 and 857 ng/cigarette, respectively). The amounts of NAB and NAT ranged from 125 to 783 ng/cigarette. It was noted that, with the ISO/FTC machine-smoking method, NNK was the predominant TSNA in sidestream smoke, while NNN was predominant in mainstream smoke. The data obtained in the Massachusetts Benchmark Study (Borgerding *et al*., 2000) showed that, under more intense smoking patterns that included partial vent blocking, American blend cigarettes released 50.7–96.7 ng/cigarette NNK, 69.8–115.2 ng/cigarette NNN, 11.9–17.8 ng/cigarette NAB and 38.4–73.4 ng/cigarette NAT (Table 6). With more intense puffing and shorter smouldering time, lower amounts of NNK than NNN were released into the ambient air.

Emissions of TSNA from sidestream smoke are significantly lower when cigarettes are smoked using a more intense machine-smoking protocol compared with the standard ISO/FTC method. The explanation for this is that the physical and chemical processes that occur in a burning cigarette change with changing puffing intensity. Moreover, there is less time between puffs to allow for tobacco combustion in the smoldering phase and release of TSNA in ambient air.

For NNK only, the yields in sidestream smoke measured by the Health Canada method were up to 2.6-fold higher than those measured by the ISO method, but only for the ‘extra light’ and ‘ultra light’ brands (Government of British Columbia, 2002).

1.3.6 *Other smoked tobacco products*

(*a*) *Cigars*

Levels of NNK, NNN and NAT in cigars from the Netherlands were 2850–4250, 6750–53 000 and 4560–20 400 ng/g dry tobacco wt, respectively (Ohshima *et al*., 1985).

One Indian cigar brand contained 8900 ng/g NNK, 25 000 ng/g NNN and 13 700 ng/g NAT fresh tobacco wt (Nair *et al.*, 1989).

Under standard ISO/FTC machine-smoking conditions, the levels of NNK in the mainstream smoke of premium cigars were 17 times higher than those of medium-yield cigarettes; NNN levels were 22.4 times higher (931 versus 41.5 ng/unit) (Rickert & Kaiserman, 1999).

Djordjevic *et al*. (1997) also reported high emissions of TSNA in the mainstream smoke of small, large and premium cigars generated by a standard ICCSS (International Committee for Cigar Smoke Study) machine-smoking method developed for cigars. Concentrations in the mainstream smoke ranged from 290 to 2490 ng/unit NNK, from 595 to 1225 ng/unit NNN and from 310 to 1145 ng/unit NAT. The NNK emissions from premium cigars exceeded the highest levels reported for cigarettes (see Table 4). Djordjevic *et al*. (1997) also explored the levels of TSNA in the mainstream smoke of small cigars using methods that mimic human smoking patterns and compared the data with those obtained by standard machine-smoking methods. Under human smoking conditions, the emissions of NNK, NNN and NAT were 1.7-fold, 2.1-fold and 1.8-fold higher, respectively, than those obtained under standard protocols.

(*b*) Bidis

Bidis originated in India and have been gaining popularity in the USA during the last decade, particularly among adolescents. The levels of preformed NNK, NNN and NAT in tobacco from *bidis* sold in India ranged from 400 to 1400 ng/g, from 6200 to 12 000 ng/g and from 9000 to 12 500 ng/g, respectively, reported as wet tobacco wt (Nair *et al*., 1989). In the mainstream smoke of these *bidis*, the levels of NNK ranged from not detected to 40 ng/cigarette, those of NNN from 11.6 to 250 ng/cigarette and those of NAT from 9.9 to 175 ng/cigarette. These concentrations were comparable with those measured in the mainstream smoke of Indian cigarettes (Nair *et al*., 1989; Pakhale & Maru, 1998).

Wu *et al*. (2004) analysed the TSNA content of tobacco filler and mainstream smoke from 14 brands of *bidis* purchased in Atlanta, GA (USA). In *bidi* tobacco filler, the levels of NNK ranged from 90 to 850 ng/g and those of NNN ranged from 150 to 1440 ng/g tobacco. These levels are slightly lower than those in typical American blended cigarettes (see Table 2) and substantially lower than those in *bidis* sold in India. In mainstream smoke from these *bidis*, the levels of NNK ranged from 8.56 to 62.3 ng/cigarette and those of NNK ranged from 2.13–25.9 ng/cigarette.

The wide variation in the TSNA levels most probably reflects the hand-rolled nature of *bidis*, which results in products that have a less homogeneous tobacco content and a wider variation in overall cigarette quality. Since *bidis* contain on average 215.3 mg tobacco versus 738 mg in commercial US cigarettes (Malson *et al*., 2001), normalization of mainstream smoke emissions of TSNA per milligram of nicotine would give more realistic information on the ultimate levels of exposure to carcinogens (King *et al*., 2007).

(*c*) Chutta *tobacco*

The levels of NNK, NNN and NAT in *chutta* tobacco were reported to be extremely high: from 12 600 to 210 300 ng/g, from 21 100 to 295 800 ng/g and from 89 200 to 686 800 ng/g, respectively, reported as fresh tobacco wt. The reverse smoker inhales both the mainstream and sidestream smoke. NNK, NNN and NAT levels in the mainstream smoke of *chutta* ranged from 150 to 2651 ng, from 289 to 1260 ng and from 431 to 1722 ng/*chutta*, respectively (Nair *et al*., 1989). Stich *et al*. (1992) reported comparable levels of NNK, NNN and NAT in the mainstream smoke of *chutta*: 274–2520, 925–3910 and 141–1300 ng/cigarette, respectively.

(*d*) *Pipe tobacco*

Ohshima *et al*. (1985) analysed the TSNA content of pipe tobacco from France, the Netherlands and the United Kingdom. The concentrations of NNK ranged from not detected to 1130 ng/g, those of NNN from 3000 ng/g to 6880 ng/g and those of NAT from 1990 to 4850 ng/g dry tobacco wt, respectively. Chamberlain *et al*. (1988) reported levels of 300 ng/g tobacco NNK and 1800 ng/g tobacco NNN in pipe tobacco from the USA. TSNA emissions in the mainstream and sidestream smoke generated by pipe smoking have not been reported to date.

1.3.7 *Secondhand tobacco smoke*

The NNN concentrations measured in a poorly ventilated office where heavy smoking of cigarettes, cigars and pipes took place ranged from not detected to 6 ng/m^3 and those of NNK from not detected to 13.5 ng/m^3 (Klus *et al*., 1992). The upper levels reported by Klus *et al*. (1992) and by Adlkofer *et al*. (1990) for ‘heavily smoked rooms’ (11 cigarettes smoked in 2 h in a 84-m^2 office) were lower than those measured inside bars, restaurants, trains, a car, an office and a smoker’s home: NNN concentrations ranged from not detected to 22.8 ng/m^3 and NNK concentrations ranged between 1.4 and 29.3 ng/m^3 (Brunnemann *et al*., 1992).

1.3.8 *Smokeless tobacco products*

The occurrence of TSNA in smokeless tobacco products is discussed extensively in the monograph on Smokeless tobacco. An international comparison of the concentration ranges in a variety of smokeless tobacco products is presented in Table 7. [TSNA concentrations are customarily expressed in micrograms per gram of dry (or wet) tobacco. To enable the comparison of the TSNA levels in cigarette and smokeless tobacco, the values in this table are expressed in nanograms per gram of dry (or wet) tobacco.] There is a very wide range of concentrations of TSNA in smokeless tobacco, which reflects the product category (e.g. chewing tobacco, moist snuff, dry snuff, new low-TSNA products), product characteristics in each category (e.g. short cut or long cut tobacco, flavourings), tobacco

Table 7. International comparison of the concentration ranges of tobacco-specific *N*-nitrosamines in smokeless tobacco products (ng/g tobacco)

Country	Type of product	Reported as[a]	NNK	NNN	NAB	NAT	References
Belgium	Chewing tobacco	Dry	130	7380	NR[b]	970[c]	Ohshima *et al.* (1985)
Canada	Moist snuff	Dry	3200–5800	50 400–79 100	4000–4800	152 000–170 000	Brunnemann *et al.* (1985)
	Chewing tobacco	Dry	240	2090	100	1580	
Denmark	Chewing tobacco	Wet	19–1900	80–1600	30	180–2900	Österdahl *et al.* (2004)
Germany	Chewing tobacco	Dry	30–300	1420–2300	30–50	330–3700[b]	Brunnemann *et al.* (1985); Tricker *et al.* (1988)
	Dry snuff	Dry Wet	580–6430 100	2390–18 750 680	NR NR	1030–7830 310	Tricker & Preussmann (1991); Österdahl *et al.* (2004)
India	Moist snuff	Wet	240	560	20	380	Stepanov *et al.* (2005)
	Chewing tobacco	Dry	130–600	470–850	30–70	300–500[b]	Brunnemann *et al.* (1985); Tricker *et al.* (1988)
		Wet	2700–6500	15 300–24 400	NR	10 000–44 600	Nair *et al.* (1989)
	Zarda	Dry	220–24 100	400–79 000	NR[b]	780–99 100[c]	Tricker & Preussmann (1988); Tricker *et al.* (1988)
		Wet NR	1070–3090	4810–19 900 6550–7360	190–1190	640–1980	Stepanov *et al.* (2005) Gupta (2004)
	Mishri	Dry	294–1100	300–6995	NR	488–14 151	Tricker *et al.* (1988); Nair *et al.* (1987)
		Wet NR	870	4210 4020–4470	150	2550	Stepanov *et al.* (2005) Gupta (2004)
	Khiwam	Dry	100–1030	2500–8950	NR[b]	1830–10 360[c]	Tricker & Preussmann (1989)
	Khaini	Dry Wet	110–5290 2340–28 400	25 800–40 000 39 400–76 900	1240–2480 3870–8830	660–18 800 4830–13 800	Stich *et al.* (1992) Stepanov *et al.* (2005)
	Gutka	Wet NR	40–430 10 680–11 510	90–1090 1870–5730	ND–50 5750–6890	10–80 6270–6530	Stepanov *et al.* (2005) Gupta (2004)
	Supari	Wet NR	ND 4900–11 580	ND 1920–2450	ND 11 580–12 580	ND 3600–4570	Stepanov *et al.* (2005) Gupta (2004)

Table 7 (contd)

Country	Type of product	Reported as[a]	NNK	NNN	NAB	NAT	References
India (contd)	Creamy snuff/ toothpaste	Wet	1310–12 500 4380–4880	2520–48 700	70–110	530–26 600	Stepanov *et al*. (2005); Nair *et al*. (1989) Gupta (2004)
	Snuff for inhalation	Wet	245 500	1 356 000	NR	1 857 000	Nair *et al.* (1989)
	Toothpowder	Wet	ND	ND–40	ND	ND	Stepanov *et al*. (2005)
	Tuibur	NR		19 650–20 120			Gupta (2004)
	Other tobacco	Wet	80–2610	1740–19 200	12–1570	350–11 900	Stepanov *et al*. (2005)
Norway	Moist snuff	Wet	3300	21 000	1700	13 000	Österdahl *et al*. (2004)
South Africa	Low-TSNA moist snuff	Dry	270–290	1050–2070	90–110	890–1520	Brunnemann *et al.* (2004)
Sudan	*Toombak*	Dry	188 000–7 870 000	141 000–3 080 000	13 900–2 370 000	20 000–290 000	Idris *et al*. (1991); Prokopczyk *et al*. (1995)
Sweden	Moist snuff	Dry	190–2950	1120–154 000	40–1700	900–21 400[c]	Brunnemann *et al*. (1985); Ohshima *et al*. (1985); Tricker *et al*. (1988); Hoffmann *et al*. (1991); Tricker & Preussmann (1991); Brunnemann & Hoffmann (1992); Djordjevic *et al*. (1993a); Connolly (2001)
		Wet	190–1300	490–4400	30–170	320–3500	Österdahl & Slorach (1988); Österdahl *et al.* (2004)
	Low-TSNA moist snuff	Wet	30–360	150–2300	10–130	60–980	Österdahl *et al*. (2004); Stepanov *et al*. (2006a)
	Chewing tobacco	Wet	10–460	700–1700	ND	1100–2100	Österdahl *et al*. (2004)
Thailand	Chewing tobacco	Dry	100	500	NR[b]	500[c]	Tricker *et al*. (1988)

Table 7 (contd)

Country	Type of product	Reported as[a]	NNK	NNN	NAB	NAT	References
United Kingdom	Moist snuff	Dry	400–13 000	1100–52 000	86	2000–6500[c]	Hoffmann *et al*. (1988); Brunnemann & Hoffmann (1992)
	Chewing tobacco	Dry	300	900	NR	1500[c]	Tricker *et al*. (1988)
	Dry snuff	Dry	580–4300	2390–16 000	NR	1030–7830	Tricker & Preussmann (1991); Brunnemann & Hoffmann (1992)
		Wet	260	1800	NR	820	Österdahl *et al*. (2004)
USA	Moist snuff	Dry	ND–18 000	ND–147 000	20–10 670	240–339 000	Brunneman *et al*. (1985); Ohshima *et al*., 1985; Adams *et al*. (1987b); Brunnemann *et al*. (1987a,b); Chamberlain *et al*. (1988); Tricker *et al*. (1988); Andersen *et al*. (1989); Djordejvic *et al*. (1989a); Hoffmann *et al*. (1991); Brunnemann & Hoffmann (1992); Prokopczyk *et al*. (1992); Djordjevic *et al*. (1993a); Hoffmann *et al*. (1995); Prokopczyk *et al*. (1995); Connolly (2001); Brunnemann *et al*. (2002, 2004)
		Wet	60–13 000	710–63 000	14–2800	240–83 000	Österdahl *et al*. (2004); Stepanov *et al*. (2006a)
	Low-TSNA moist snuff	Wet	32–33	620–640	17–18	310–320	Stepanov *et al*. (2006a)

Table 7 (contd)

Country	Type of product	Reported as[a]	NNK	NNN	NAB	NAT	References
USA (contd)	Chewing tobacco	Dry	ND–1100	670–6500	20–140	670–12 400	Brunnemann *et al*. (1985); Chamberlain *et al*. (1988); Andersen *et al*. (1989); Djordjevic *et al*. (1989a); Brunnemann & Hoffmann (1992)
		Wet	80–110	250–1100	20	150–940	Österdahl *et al*. (2004)
	Dry snuff	Dry	880–84 400	9370–116 100	520–1530	11 200–238 800[c]	Adams *et al*. (1987); Brunnemann *et al*. (1987a); Andersen *et al*. (1989); Djordjevic *et al*. (1989a)
	Compressed tobacco lozenges	NR	37–43	19–56	7–8	12–17	Stepanov *et al*. (2006a)
Uzbekistan	*Naswar*	Dry	20–130	120–520	8–30	32–300	Brunnemann *et al*. (1985)

NAB, *N*′-nitrosoanabasine; NAT, *N*′-nitrosoanatabine; ND, not detected; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNN, *N*′-nitrosonornicotine; NR, not reported

[a] Reported as ng/g dry wt (dry) or wet wt (wet) tobacco

[b] The upper value reported as NAT also includes NAB.

[c] The upper value includes NAB + NAT.

blend, tobacco curing and manufacturing technologies, ageing, country of origin, year of production and analytical methods.

Because of the prohibition of the marketing of moist snuff in the European Union except in Sweden and in the EFTA (European Free Trade Association) country Norway (see the monograph on Smokeless tobacco, Section 1.5.3), data on the TSNA content of smokeless tobacco products marketed in the European countries beyond 1992 are limited. Chewing tobacco contained the lowest amounts of TSNA: NNK ranged from undetectable levels (USA) to 1900 ng/g wet wt (Denmark); NNN ranged from 80 ng/g wet wt (Denmark; Österdahl *et al.*, 2004) to 7380 ng/g dry wt (Belgium; Ohshima *et al.*, 1985); NAB ranged from undetectable levels to 140 ng/g dry wt (USA) and NAT ranged from 150 ng/g wet wt to 12 400 ng/g dry wt (USA).

The levels of NNK, NNN, NAB and NAT in moist snuff were, in general, substantially higher than those in chewing tobacco; upper concentrations reached 18 000 ng/g dry tobacco NNK (USA; Connolly, 2001), 154 000 ng/g dry tobacco NNN (Sweden; Ohshima *et al.*, 1985), 10 670 ng/g dry tobacco NAB (USA; Brunnemann & Hoffmann, 1992) and 339 000 ng/g dry tobacco NAT (USA; Ohshima *et al.*, 1985). Levels in Indian moist snuff were surprising low (Stepanov *et al.*, 2005).

High levels of TSNA in dry snuff were also measured in the USA: NNK, up to 84 400 ng/g dry tobacco; NNN, up to 116 100 ng/g dry tobacco; NAB, up to 1530 ng/g dry tobacco and NAT, up to 238 000 ng/g dry tobacco. In Indian snuff used for inhalation, TSNA levels were even higher: 245 000 ng/g fresh tobacco NNK, 1 356 000 ng/g fresh tobacco NNN and 1 875 000 ng/g fresh tobacco NAT (Nair *et al.*, 1989).

Although there has been a decline in the concentrations of TSNA in smokeless tobacco products in Sweden and the USA since the 1980s (Djordjevic *et al.*, 1993b; Brunnemann *et al*., 2004; Österdahl *et al*., 2004), the trend may not apply to other products and countries. For example extremely high levels (in milligrams) of TSNA were measured in *toombak* (Idris *et al*., 1991; Prokopczyk *et al*., 1995; Idris *et al*., 1998): NNK, up to 7 870 000 ng/g dry tobacco; NNN, up to 3 085 000 ng/g dry tobacco; NAB, up to 237 000 ng/g dry tobacco and NAT, up to 290 000 ng/g dry tobacco. In contrast, very low levels of TSNA have been reported in *naswar*: NNK, 130 ng/g dry tobacco; NNN, up to 520 ng/g dry tobacco; NAB, 30 ng/g dry tobacco; and NAT, 300 ng/g dry tobacco.

In recent years, some manufacturers of smokeless tobacco products have used novel tobacco curing and processing technologies to produce moist snuff with lower TSNA. These products are marketed in Norway, South Africa, Sweden, the USA and other countries (Connolly, 2001; Brunnemann *et al*., 2004; Österdahl *et al*., 2004; Stepanov *et al*., 2006a; McNeill *et al*., 2007). Levels of NNK and NNN in moist snuff produced by the new manufacturing process (Gothia, 2004) have been reported to be up to 45 times lower than those in leading products manufactured under standard process (Connolly, 2001). In Sweden, all moist snuff brands on the market in 2002 contained low levels of TSNA. NNN concentrations in moist snuff have decreased consistently from 1983 to 2002 in this country (Österdahl *et al*., 2004). However, low-TSNA Swedish *snus*, purchased both in Sweden and the USA between 2003 and 2005, contained up to 360 ng/g wet

tobacco NNK, up to 2300 ng/g wet tobacco NNN, up to 130 ng/g wet tobacco NAB and up to 980 ng/g wet tobacco NAT (Stepanov *et al*., 2006a). Since the values were not adjusted for the moisture content, the concentrations may be even higher. Other new smokeless tobacco products (e.g. compressed tobacco lozenges such as Ariva®, Stonewall, Hard Snuff) contain even lower concentrations of NNK, NNN, NAB and NAT (Table 7).

In India, smokeless tobacco products account for over one-third of all tobacco used (see the monograph on Smokeless tobacco). Traditional forms of smokeless tobacco include betel quid with tobacco, tobacco with lime and tobacco tooth powder but a variety of new products are gaining popularity (Gupta & Ray, 2003). Table 7 presents an overview of TSNA concentrations in a variety of Indian products. The highest levels of NNK, NNN, NAB and NAT were reported in *zarda* and *khaini* (Tricker & Preussmann, 1988; Tricker *et al*., 1988; Stepanov *et al*., 2005). Other products also contain high levels of TSNA (Gupta, 2004; Stepanov *et al*., 2005). In contrast, *gutka*, moist snuff and chewing tobacco contain significantly lower levels of TSNA (Stepanov *et al*., 2005). Some tobacco products consumed in India, such as *supari* and toothpowder, did not contain quantifiable levels of TSNA (Stepanov *et al*., 2005). Brown and black *mishri*, a half-burnt tobacco product commonly used as a dentifrice in some parts of India, were reported to contain significantly higher amounts of TSNA compared with unburnt tobacco: NNK, up to 1100 ng/g dry wt; NNN, up to 6995 ng/g dry wt; and NAT, up to 14 150 ng/g dry wt (Nair *et al.*, 1987; Tricker *et al.*, 1988). In addition to TSNA, *mishri* contains high concentrations of benzo[*a*]pyrene (from 27 to 119 µg/g; Nair *et al.*, 1987).

Hoffmann *et al*. (1995) compared the concentrations of TSNA between two major categories of moist snuff that contain high and low levels of unprotonated nicotin (Table 8). In the high-level unprotonated nicotine brands, concentrations were in the range of: NNK,

Table 8. The levels of tobacco-specific *N*-nitrosamines (TSNA) in five leading brands of moist snuff purchased in the USA, 1994

Product	TSNA (µg/g dry wt)				
	NNK	NNN	NAB	NAT	Total TSNA
Skoal Bandits Straight[a]	0.92 ± 0.26	5.09 ± 1.03	0.13 ± 0.03	2.05 ± 0.60	8.19 ± 1.72
Hawken Wintergreen[a]	0.23 ± 0.04	3.07 ± 0.3	0.13 ± 0.02	0.63 ± 0.15	4.08 ± 0.44
Skoal Original Fine Cut Wintergreen[b]	1.25 ± 0.13	8.18 ± 1.33	0.37 ± 0.09	5.10 ± 1.01	14.90 ± 2.50
Copenhagen Snuff[b]	1.89 ± 0.62	8.73 ± 1.44	0.50 ± 0.12	6.13 ± 1.02	17.24 ± 2.97
Kodiak Wintergreen[b]	0.55 ± 0.15	6.3 ± 1.06	0.32 ± 0.10	3.79 ± 1.25	10.96 ± 2.44

From Hoffmann *et al.* (1995)

NAB, *N*′-nitrosoanabasine; NAT, *N*′-nitrosoanatabine; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNN, *N*′-nitrosonornicotine

[a] Brands with low levels of unprotonated nicotine

[b] Brands with high levels of unprotonated nicotine

550–1890 ng/g dry tobacco; NNN, 6300–8730 ng/g dry tobacco; NAB, 320–500 ng/g dry tobacco; and NAT, 3790–6130 ng/g dry tobacco. In the low-level unprotonated nicotine brands, concentrations were in the range of: NNK, 230–920 ng/g dry tobacco; NNN, 3070–5090 ng/g dry tobacco; NAB, 130 ng/g dry tobacco; and NAT, 630–2050 ng/g dry tobacco. Thus, the three leading snuff brands in the USA (Copenhagen, Skoal Fine Cut and Kodiak) that made up 92% of the market in 1995 not only had a high pH and contained high levels of nicotine and unprotonated (free) nicotine, but also contained high concentrations of carcinogenic TSNA in comparison with the moist snuff brands that ranked fourth and fifth — Hawken and Skoal Bandits (3% of the US market).

A high-pH (7.99) and high-moisture (52.7%) moist snuff brand purchased in the USA in 2000 contained 2500 ng/g NNK, 15 400 ng/g NNN, 1200 ng/g NAB and 18 500 ng/g NAT, reported as dry tobacco wt; in contrast, a low-pH (5.84) and low-moisture (24%) moist snuff brand contained 500 ng/g NNK, 3100 ng/g NNN, 200 ng/g NAB and 800 ng/g NAT (Brunnemann *et al.*, 2002). TSNA levels in low-moisture brands are generally more consistent over time while they fluctuate dramatically in high-moisture brands.

In general, TSNA levels per unit dose are higher in smokeless tobacco compared with the levels in the mainstream cigarette smoke as determined by the ISO/FTC machine-smoking method (see Tables 4 and 7). Even in Swedish *snus*, levels of TSNA are comparable with those in cigarette smoke (e.g. the highest reported ISO/FTC NNN yield in the mainstream smoke was 1353 ng/cigarette versus 2300 ng/g *snus*; Stepanov *et al*., 2006a).

1.4 Biomonitoring in saliva, urine and other tissues

Analysis of TSNA and their metabolites in saliva, urine and blood has proven to be extremely useful in estimating human exposure to these carcinogens. While studies of levels in saliva and blood have been somewhat limited, there is extensive literature of urinary NNAL and NNAL-glucuronides (NNAL-Gluc), metabolites of NNK (see Section 4.1), in smokers, smokeless tobacco users and nonsmokers exposed to secondhand smoke. Studies relevant to the toxicokinetics (absorption, distribution, metabolism and excretion) of TSNA in humans and to the mechanisms of carcinogenesis by these compounds are discussed in Section 4. This section presents data that relate tobacco exposure to TSNA and their metabolites. Levels of NNK, NNN, NAB and NAT in the saliva of tobacco users are summarized in Table 9. Only studies published since the previous evaluation (IARC, 1985) have been included.

1.4.1 *4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and its metabolites*

(*a*) *NNK and its metabolites in saliva*

NNK has been detected in the saliva of snuff dippers, users of *khaini*, of *gudhaku* chewers of betel quid with tobacco and users of *toombak* (Table 9; Nair *et al.*, 1985; Bhide *et al.*, 1986; Österdahl & Slorach, 1988; Idris *et al.*, 1992; Hoffmann *et al.*, 1994). Levels in snuff-dippers ranged from not detected to 201 ng/g saliva and those in *khaini* users were

Table 9. Concentrations of NNK, NNN, NAB and NAT in the saliva of users of various forms of tobacco

Reference	Country of study	Tobacco product	Unit	Mean concentration (range) or range			
				NNK	NNN	NAB	NAT
Nair *et al.* (1985)	India	Betel quid with tobacco	ng/mL saliva	0.34 (0–2.3)	7.5 (1.6–14.7)	–	4.8 (1.0–10.9)
		Chewing tobacco		ND	33.4 (16.5–59.7)		29.8 (13.5–51.7)
		Cigarette		ND	ND	–	ND
Bhide *et al.* (1986)	India	Betel quid with tobacco	ng/g saliva	–	3–85.7	ND–40	–
		Mishri		ND	23.7 (14.3–43.5)	ND	–
		Khaini		ND–28.5	91.9 (10.0–430)	–	ND–133.0
Brunnemann *et al.* (1987c)	Canadian Inuits	Snuff	ng/g saliva	56 (ND–201)	980 (115–2601)	–	1318 (123–4560)
Österdahl & Slorach (1988)	Sweden	Snuff	ng/g saliva	ND–16	3–140	–	4–85
Idris *et al.* (1992)	Sudan	*Toombak*	ng/mL saliva	ND–6689	582–20988	46–1944	ND–471
Stich *et al.* (1992)	India	*Khaini*	ng/mL saliva	ND–180	150–1580	13–90	86–690
		Gudhaku	ng/mL saliva	ND–10	15–88	1–15	9–55
		Chutta (reverse smoking)	ng/g saliva	–	45–5890	–	ND–1880

NAB, *N*′-nitrosoanabasine; NAT, *N*′-nitrosoanatabine; ND, not detected; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNN, *N*′-nitrosonornicotine

up to 180 ng/g saliva. The highest levels were found in *toombak* users (up to 6690 ng/g saliva). Levels of NNK were generally 10–100 times greater in the saliva of *toombak* users than in users of other types of smokeless tobacco (Idris *et al.*, 1992; Hoffmann *et al.*, 1994).

NNAL, a metabolite of NNK, was also detected in the saliva of *toombak* users at levels as high as 3270 ng/g saliva (Idris *et al.*, 1992).

(*b*) *NNK and its metabolites in cervical mucus and pancreactic juice*

NNK was detected in 16 samples of cervical mucus from 15 women who were smokers at concentrations of 11.9–115 ng/g mucus (two samples were collected from one smoker at different times) and in nine of 10 samples from nonsmokers at concentrations of 4.1–30.8 ng/g mucus; the concentrations of NNK in specimens from cigarette smokers were significantly higher than in those obtained from nonsmokers (Prokopczyk *et al*., 1997).

NNK was detected in 15/18 samples of pancreatic juice from smokers at concentrations of 1.4–604 ng/mL and in six of nine samples from nonsmokers (range of concentrations, 1.13–97 ng/mL); the levels were significantly higher in smokers than in nonsmokers. NNAL was present in 11/17 samples from smokers and in three of nine samples from nonsmokers (Prokopczyk *et al.*, 2002).

(*c*) *NNAL and NNAL-Gluc in urine*

Unchanged NNK is not detected in urine (Hecht *et al.*, 1999a).

Several studies have quantified NNAL and NNAL-Gluc in human urine. Levels of total NNAL (NNAL plus NNAL-Gluc) are generally 2–4 pmol/mg creatinine in smokeless tobacco users, 1–4 pmol/mg creatinine in smokers and 0.02–0.07 pmol/mg creatinine in nonsmokers exposed to secondhand smoke. Several studies have quantified these metabolites in 24-h urine. Baseline levels of excreted NNAL and NNAL-Gluc are typically about 1 nmol NNAL/24 h and 2.2 nmol NNAL-Gluc/24 h (Hecht *et al.*, 1999a); levels of total NNAL generally reported were 6.6 nmol/24 h in smokeless tobacco users, 3–4 nmol/24 h in smokers and 0.03–0.13 nmol/24 h in nonsmokers exposed to secondhand tobacco smoke. Occasionally, however, as in the case of *toombak* users, far higher levels of total NNAL in urine have been observed.

(i) *Smokers*

The earlier literature on NNAL and NNAL-Gluc in the urine of smokers has been reviewed (Hecht, 2002; IARC, 2004). In a study of 274 smokers of cigarettes in the contemporary yield ranges of the German market and 100 nonsmokers, total NNAL levels were significantly higher in smokers than in nonsmokers (Scherer *et al.*, 2007).

Total NNAL has been shown to correlate with number of cigarettes smoked per day but the increase was not linear (Joseph *et al.*, 2005). No statistically significant differences in urinary levels of total NNAL were observed in urine samples from 175 smokers of regular, light or ultra-light cigarettes (Burns *et al.*, 2001; Harris *et al.*, 2004; Bernert *et al.*, 2005; Hecht *et al.*, 2005).

Two studies investigated the effects of reducing the number of cigarettes smoked per day on levels of NNAL and NNAL-Gluc. Decreases in levels of NNAL, NNAL-Gluc and total NNAL were observed, but these were generally modest, and were always proportionally less than the reduction in the number of cigarettes smoked per day (Hurt *et al.*, 2000; Hecht *et al.*, 2004a).

Levels of total NNAL were significantly reduced in smokers who switched from their customary brand to the Omni cigarette (a cigarette with reduced levels of NNK) and were significantly lower than those in smokers who stopped with the aid of a nicotine patch (Hatsukami *et al.*, 2004).

A comparison of total NNAL in the urine of Caucasians and African–Americans demonstrated that mean concentrations were greater in African–American men for each cigarette smoked; no difference was seen in women (Muscat *et al.*, 2005).

(ii) *Nonsmokers exposed to secondhand tobacco smoke*

Levels of total NNAL in the urine of nonsmokers exposed to secondhand tobacco smoke are typically about 1–5% of those in smokers (Hecht, 2002; IARC, 2004). In more recent studies, total NNAL was quantified before and after a 4-h visit to a gambling casino where smoking was allowed (Anderson *et al.*, 2003) and in nonsmokers who worked in restaurants and bars that allow smoking (Tulunay *et al.*, 2005). Both studies showed significant increases in urinary levels of total NNAL after exposure to secondhand smoke.

Levels of total NNAL in the urine of 144 infants (< 1 year old) averaged 0.083 ± 0.200 pmol/mL urine; the mean number of cigarettes smoked per week by any family member in the home or car when the infant was present was significantly higher in the families of children with detectable levels of NNAL compared to those with undetectable levels. The levels of NNAL detected in the urine of these infants was higher than those in most other field studies of exposure to secondhand tobacco smoke (Hecht *et al.*, 2006).

In another study, total NNAL was detected in 69/80 urine samples from Moldovan children. The mean level (0.09 ± 0.077 pmol/mL) was comparable with those observed in previous studies of secondhand smoke exposure (Stepanov *et al.*, 2006b).

(iii) *Smokeless tobacco users*

Snuff dippers/tobacco chewers in the USA excreted 6.6 nmol/24 h total NNAL (NNAL plus NNAL-Gluc) in urine (Hecht *et al.*, 2002). In one study of snuff dippers and tobacco chewers, urinary excretion of total NNAL averaged 4.4 pmol/mg creatinine. Levels in 23 snuff-dippers (5.9 pmol/mg creatinine) were significantly higher than those in 13 tobacco chewers (2.1 pmol/mg creatinine) (Kresty *et al.*, 1996).

Seven *toombak* users excreted an average of 1270 pmol/mL urine total NNAL, which was approximately 300 times that excreted by snuff-dippers/chewers (Murphy *et al.*, 1994).

Among snuff dippers in the USA, total daily dip duration, total daily dipping time and number of dips per day were significantly correlated with levels of total NNAL (Lemmonds *et al.*, 2005). Levels of total NNAL correlated with the number of tins used per day in one study (Hecht *et al.*, 2002) but not in another (Lemmonds *et al.*, 2005). Total levels of NNAL

were significantly lower in users of smokeless tobacco after they switched to Swedish snuff or to nicotine patch; the overall mean level of total NNAL among subjects who used a nicotine patch was significantly lower than that among those who used snuff (Hatsukami *et al.*, 2004).

Levels of NNAL and NNAL-Gluc were quantified in the urine of 420 smokers and 182 smokeless tobacco users (Hecht *et al.*, 2007). Levels of total NNAL/mL urine and levels of total NNAL/mg creatinine, adjusted for age and sex, were significantly higher in smokeless tobacco users than in smokers ($p < 0.001$).

(*d*) *Total NNAL in blood*

A liquid chromatography–electrospray ionization tandem mass spectrometry method was developed for the analysis of total NNAL in plasma. Levels averaged 42 ± 22 fmol/mL in 16 smokers; total NNAL was not detected in the plasma of nonsmokers. Levels were 1–2% of those found in urine (Carmella *et al.*, 2005, 2006).

(*e*) *Total NNAL in toenails*

Total NNAL, nicotine and cotinine were analysed in human toenails by liquid chromatography–electrospray ionization tandem mass spectrometry in 35 smokers. Mean total NNAL was 0.41 ± 0.67 pg/mg toenail (Stepanov *et al.*, 2006c).

1.4.2 N′-*Nitrosonornicotine (NNN)*

(*a*) *Saliva*

Formation of additional quantities of NNN by the reaction of salivary nitrite with nicotine or nornicotine during the oral use of snuff or during tobacco chewing has been implied from in-vitro studies (Hoffmann & Adams, 1981).

NNN has been detected in the saliva of snuff dippers (Brunnemann *et al.*, 1987c; Österdahl & Slorach, 1988; Hoffmann *et al.*, 1994), chewers of betel quid with tobacco (Nair *et al.*, 1985; Bhide *et al.*, 1986), users of *khaini* (Bhide *et al.*, 1986; Stich *et al.*, 1992), users of *gudakhu* (Stich *et al.*, 1992), users of *mishri* (Bhide *et al.*, 1986), reverse smokers of *chutta* (Stich *et al.*, 1992) and users of *toombak* (Idris *et al.*, 1992) (Table 9). The exceptionally high levels of NNN in the saliva of *toombak* users reflects the unusually high concentrations of NNN (mg/g) in this product (Idris *et al.*, 1991).

(*b*) *Urine*

Levels of NNN in the urine of 14 smokers ranged from not detected to 0.43 pmol/mg creatinine (mean ± standard deviation (SD), 0.086 ± 0.12 pmol/mg) and those of NNN-*N*-glucuronide (NNN-*N*-Gluc) ranged from not detected to 0.36 pmol/mL creatinine (mean ± SD, 0.096 ± 0.11 pmol/mL). The corresponding values in 11 smokeless tobacco users were 0.03–0.58 pmol/mg creatinine (mean ± SD, 0.25 ± 0.19 pmol/mg) and 0.091–0.91 pmol/mg creatinine (mean ± SD, 0.39 ± 0.27 pmol/mg) (Stepanov & Hecht, 2005).

(*c*) *Pancreatic juice*

NNN was found in two of 17 samples of pancreatic juice from smokers (68 and 242 ng/mL) (Prokopczyk *et al*., 2002).

1.4.3 N′-*Nitrosoanabasine (NAB)*

(*a*) *Saliva*

NAB was detected in the saliva of chewers of betel quid with tobacco (Bhide *et al*., 1986), *toombak* (Idris *et al.*, 1992), *khaini* (Stich *et al.*, 1992) and *gudhaku* (Stich *et al.*, 1992) (Table 9).

(*b*) *Urine*

NAB and NAB-*N*-glucuronide (NAB-*N*-Gluc) have been found in the urine of smokers and smokeless tobacco users. Levels in 14 smokers ranged from not detected to 0.019 pmol/mg creatinine (mean ± SD, 0.003 ± 0.006 pmol/mg) NAB and from not detected to 0.14 pmol/mg creatinine (mean ± SD, 0.038 ± 0.039 pmol/mg) NAB-*N*-Gluc. Corresponding levels in 11 smokeless tobacco users ranged from not detected to 0.11 pmol/mg creatinine (mean ± SD, 0.037 ± 0.034 pmol/mg) and 0.028–0.44 pmol/mg creatinine (mean ± SD, 0.19 ± 0.16 pmol/mg), respectively (Stepanov & Hecht, 2005).

1.4.4 N′-*Nitrosoanatabine (NAT)*

(*a*) *Saliva*

NAT has been detected in the saliva of snuff dippers (Brunnemann *et al*., 1987c; Österdahl & Slorach, 1988; Hoffmann *et al.*, 1994), *toombak* users (Idris *et al.*, 1992), *khaini* tobacco users (Bhide *et al.*, 1986; Stich *et al.*, 1992), *gudhaku* users (Stich *et al.*, 1992) and users of betel quid with tobacco (Nair *et al.*, 1985) (Table 9).

(*b*) *Urine*

NAT and NAT-*N*-glucuronide (NAT-*N*-Gluc) were detected in the urine of smokers and smokeless tobacco users. Levels in 14 smokers ranged from not detected to 0.31 pmol/mg creatinine (mean ± SD, 0.067 ± 0.104 pmol/mg) NAT and from not detected to 0.43 pmol/mg creatinine (mean ± SD, 0.12 ± 0.11 pmol/mg) NAT-*N*-Gluc. Corresponding levels in 11 smokeless tobacco users were 0.020–0.15 pmol/mg creatinine (mean ± SD, 0.069 ± 0.046 pmol/mg) and 0.08–2.78 pmol/mg creatinine (mean ± SD, 1.36 ± 1.06 pmol/mg), respectively (Stepanov & Hecht, 2005).

2. Studies of Cancer in Humans

No data were available to the Working Group.

3. Studies of Cancer in Experimental Animals

Volumes of data exist on tumour development in various animal models that have been exposed to TSNA by various modes of administration. Not all of these studies are included in the monograph. Studies presented here are considered to be pivotal for the establishment of the carcinogenicity of NNK, NNN and NNAL. Animals studies on these substances have been reviewed comprehensively (Hecht, 1998). Studies of NAB and NAT are also reviewed although the number of studies are fewer.

3.1 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) (Table 10)

The carcinogenicity of NNK in experimental animals has been evaluated previously (IARC, 1985).

3.1.1 *Intraperitoneal administration*

Mouse

Groups of 25 female strain A/J mice, 6–8 weeks of age, received thrice-weekly intraperitoneal injections of 0.1 mL of a 1% solution of NNK [purity not specified] in trioctanoin for a total of 22 injections (total dose, 22 mg or 0.11 mmol per mouse) and were observed for 30 weeks after the final injection. Controls consisted of groups of 25 of untreated mice, vehicle controls and positive (urethane-treated) controls. When animals were killed, lungs were examined macroscopically for total lesions and microscopically for histological type. The Student's *t*-test was used to determine statistical significance. In untreated controls, 1/25 animals developed lung tumours at a multiplicity of 0.04 ± 0.20 tumours per mouse; in the vehicle controls, 5/24 animals developed lung tumours at a multiplicity of 0.2 ± 0.41 tumours per mouse. Of the NNK-treated animals, 20/23 developed lung tumours with a multiplicity of 2.61 ± 1.85 tumours per mouse ($p < 0.05$ compared with vehicle controls). Tumours were described as adenomas (Hecht *et al*., 1978).

Groups of 25 female strain A/J mice, 6–8 weeks of age, received thrice-weekly intraperitoneal injections of 0.1 mL of a 1% solution of NNK (> 99% pure) suspended in trioctanoin for 7.3 weeks (22 injections; total dose, 0.11 mmol/mouse) and were held for 30 weeks after the last injections. Untreated and vehicle-treated animals served as controls.

Table 10. Summary of reports of tumours induced in experimental animals by NNK and NNN

Compound/ species	Lung	Nasal cavity	Oral cavity	Trachea	Oeso-phagus	Fore-stomach	Pancreas	Liver	Adrenal gland	Skin
NNK										
Mouse	x					x		x		x
Rat	x	x	x[a]				x	x		
Hamster	x	x		x			x[c]		x[c]	
Mink	x	(x)								
NNN										
Mouse	x					x				
Rat		x	x[b]		x					
Hamster		x		x						
Mink		(x)								

NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNN, *N*′-nitrosonornicotine
[a] In combination with NNN
[b] In combination with NNK
[c] In progeny

Lung tumours were counted macroscopically at the time of sacrifice and were fixed in 10% formalin for histological evaluation. The Student's *t*-test was used to determine statistical significance. Of the NNK-treated mice, 23/23 developed 865 lung tumours (412 carcinomas) with a multiplicity of 37.6 ± 11.8 tumours per mouse ($p < 0.0001$ compared with vehicle controls). Six treated mice had tumours other than lung adenomas: three hepatocellular adenomas, two hepatocellular carcinomas and one squamous-cell papilloma of the nasal cavity. In untreated animals, 10/25 animals developed lung adenomas with a multiplicity of 0.6 ± 0.9 adenomas per surviving mouse whereas 4/25 trioctanoin controls developed lung adenomas with a multiplicity of 0.2 ± 0.5 adenomas per mouse (Castonguay *et al*., 1983a).

In a study in which NNK and several structural analogues of NNK and NNN were examined, groups of 30 female strain A/J mice, 6–8 weeks of age, received thrice-weekly intraperitoneal injections of NNK (total dose, 20 μmol/mouse) in 0.2 mL saline for 7 weeks. NNK was synthesized and considered pure by high-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC). Mice were held for 30 weeks after the last injection. The Student's *t*-test was used to determine statistical significance. Saline control mice had a lung tumour incidence of 40% (12/30), with a tumour multiplicity of 0.5 ± 0.7. NNK induced a 100% (30/30) incidence of lung tumours with a multiplicity of 7.2 ± 3.4 tumours per mouse ($p < 0.0001$) (Hecht *et al*., 1988a). [The Working Group noted that the number of surviving animals was not listed, only macroscopic examination was carried out and that histological confirmation was not presented.]

Groups of 25 female strain A/J mice mice [age unspecified] received thrice-weekly intraperitoneal injections of NNK (99% pure by HPLC and gas chromatography–mass spectrometry) in 0.1 mL saline for a total of 20 injections (total dose, 20 μmol/mouse). Animals were held for 30 weeks after the final injection and statistical significance was

evaluated using Student's *t*-test. Controls consisted of animals injected over the same schedule with saline only. NNK induced a lung tumour incidence of 100% (25/25) and a multiplicity of 15.7 ± 4.1 tumours per mouse (saline control: 20% incidence (5/25); tumour multiplicity of 0.20 ± 0.40 tumours per mouse; [$p < 0.0001$]). Twenty-three treated mice had adenomas only and two had both adenomas and adenocarcinomas (Rivenson *et al*., 1989).

Female strain A/J mice, 5 weeks of age, were maintained on an AIN-76A or NIH-07 diet. At 7 weeks of age, groups of 15 mice (weighing 18.7 ± 0.07 g) received a single intraperitoneal injection of 0 (saline control), 2.5, 5 or 10 μmol NNK (> 99% pure) in 0.1 mL saline and were killed at semi-monthly intervals between 3 and 7 months after injection. Lung tumours were enumerated for each period and statistical significance was evaluated by ANOVA followed by Newman-Kuels' range test and chi-squared test. Selected tumours were confirmed by histopathology. Lung tumour incidence eventually reached 100% in nearly all NNK-treated groups. Lung tumour multiplicity became maximal at 3.5 months, with no significant increase between 3.5 and 7 months. A dose–response was seen for tumour multiplicity at 3.5 months: 0 tumour per mouse with saline alone, 1.0 ± 0.2 tumours per mouse with 2.5 μmol NNK, 3.7 ± 0.7 tumours per mouse with 5 μmol and 9.6 ± 0.8 tumours per mouse with 10 μmol. In mice given 10 μmol NNK, lung tumour multiplicity at 7 months did not increase over that found at 3.5 months. Final overall tumour multiplicities with 5 μmol and 2.5 μmol NNK were significantly lower than those with 10 μmol (Hecht *et al*., 1989).

To characterize and quantify lung lesions and their progression, groups of 15 female strain A/J mice, 6 weeks of age, received a single intraperitoneal injection of 100 mg/kg bw NNK (99% pure) suspended in trioctanoin and were maintained on an NIH-07 diet. Mice were killed starting at 14 weeks after injection and every 4 weeks thereafter up to 54 weeks. At 14 weeks, 100% of the lesions were hyperplasias; at 34 weeks the types and frequencies of lesions ranged from hyperplasia (57%), adenoma from hyperplasia (18%), adenoma (14%), carcinoma from adenoma (0%), carcinoma (8%) to microcarcinoma (3%). By 54 weeks, 13% of hyperplasia, 4% of adenoma from hyperplasia, 29% of adenoma, 18% of carcinoma from adenoma, 38% of carcinoma and 0% of microcarcinoma were observed (Belinsky *et al*., 1992). [The Working Group noted that the percentages at 54 weeks added up to 102%.]

Two groups of female strain A/J mice, 6–8 weeks of age, received thrice weekly intraperitoneal injection of a total dose of 5 μmol (30 mice) or 20 μmol (20 mice) NNK in 0.1 mL saline for 7 weeks. After 30 weeks, animals were killed and lung adenomas were enumerated. Statistical analysis of variance (nested model) followed by Scheffe's test for multiple comparison of means was conducted. A group of 30 negative control mice was injected with saline alone. In controls, tumour incidence was 26.7% (8/30) and tumour multiplicity was 0.27 ± 0.58 tumours per mouse; in NNK-treated mice, the incidence of tumours was 76.7% (23/30; $p < 0.001$) and 100% (20/20; $p < 0.001$) and tumour multiplicity was 1.6 ± 1.2 and 9.2 ± 6.3 tumours per mouse, in the low- and high-dose groups, respectively (Amin *et al*., 1996).

3.1.2 *Intravesicular administration*

Rat

Groups of 12 female Fischer 344 rats, 10 weeks of age, received twice-weekly instillations of 0.2 mL of a solution of 11 mg NNK (> 98% purity) dissolved in ethanol and diluted in sterile water for 30 weeks (total dose, 1.5 mmol) into the urinary bladder after excreting the residual urine. At a median of 70 weeks, no bladder tumours were observed in rats of either control or experimental groups, but 33% (4/12) of the rats exposed intravesically to NNK had liver tumours and 42% (5/12) had lung tumours [no further histopathological details were provided]. No liver or lung tumours were reported in controls (Lijinsky *et al*., 1991).

3.1.3 *Administration in the drinking-water*

(*a*) *Mouse*

Groups of male BALB/c mice [initial number unspecified], 8 weeks of age, were untreated or received 1 mg NNK in distilled water deposited on the tongue thrice weekly (total dose, 22 mg NNK); groups of male Swiss mice [initial number unspecified], 8 weeks of age, were untreated or received 0 (vehicle control) or 1 mg NNK in distilled water deposited on the tongue thrice weekly (total dose, 22 mg NNK). Animals were killed at 22 months or when moribund. Tumours were observed in 10/13 (77%) NNK-treated Swiss mice (nine lung adenomas, one forestomach papilloma and one hepatoma) and in 11/11 (100%) NNK-treated BALB/c mice (four lung adenomas, six forestomach papillomas and one hepatoma) [the Working Group noted that these tumours were reported only for nine of the 11 mice]). Tumours [not specified] developed in 2/19 (11%) untreated Swiss mice and no tumours were observed in 14 untreated BALB/c mice or 11 Swiss mice given distilled water only (Padma *et al*., 1989a).

(*b*) *Rat*

Male Fischer 344 rats, 8 weeks of age, were given 0.0 ppm [mg/mL] (80 rats), 0.5 ppm (80 rats), 1.0 ppm (80 rats) or 5.0 ppm (30 rats) NNK (> 99% pure) in the drinking-water from 8 weeks of age until the animals were killed at 128 weeks (0.5 ppm and control) or when moribund (108–120 weeks for 1.0 and 5.0 ppm NNK). A group of 80 rats (water only) served as controls. The incidence of lung tumours in controls and at 0.5-ppm, 1.0-ppm and 5.0-ppm NNK was 6/80, 9/80, 20/80 ($p < 0.01$) and 27/30 ($p < 0.01$), respectively. In the 1.0-ppm group, most of the tumours were adenomas, whereas in the 5.0-ppm groups, most were adenocarcinomas (13/27) and adenosquamous carcinomas (9/27). The incidence of exocrine pancreatic tumours in the groups treated with 0.0, 0.5, 1.0 and 5.0 ppm NNK was 1/80, 5/80, 9/80 ($p < 0.05$) and 2/80, respectively. Tumours in the 1.0-ppm group were eight acinar adenomas and one acinar or ductal adenocarcinoma. The authors speculated that the low incidence of pancreatic tumours in the 5.0-ppm group was due to the high incidence of tumours of the lung, nasal cavity and liver, which shortened survival (Rivenson *et al*., 1988).

3.1.4 *Oral cavity swabbing*

Rat

Groups of male Fischer 344 rats, 10 weeks of age, were swabbed in the oral cavity and lips with 0.3 mL of a 0- (control) or 15-mmol solution of NNK (purity, > 99%) using a cotton swab dipped into the solution until the entire 0.3 mL was used; the cotton swab was then rinsed with 0.1 mL water and this solution was also applied. Rats were treated three times during the first week, five times over 5 days for 3 weeks and, from the 5th week onwards, twice a day for 5 days per week until termination at 71 weeks of age. The approximate total dose of NNK was 539 mg or 2.60 μmol. Statistical analysis was by Student's *t*-test. This protocol produced only one papilloma in the oral cavity of 29 rats and no tumours were observed in the oesophagus. However, significant tumour formation was found in the lungs (5/29 adenomas; 19/29 adenocarcinoma; and 4/29 adenosquamous carcinoma), the nasal cavity (13/29 papilloma or adenoma; and 2/29 carcinoma) and liver (9/29 adenoma; 3/29 carcinoma). No tumours were observed in control animals (Prokopczyk *et al*., 1991).

3.1.5 *Cheek pouch application*

Hamster

Groups of male and female Syrian golden hamsters, 8 weeks of age, received applications of 0 or 1 mg NNK in distilled water on the cheek pouch three times a week (total dose, 120 mg). Animals were killed at 22 months or when moribund. Tumours occurred in 5/9 treated hamsters (two lung adenomas, four forestomach papillomas and one hepatoma); no tumours occurred in 11 untreated hamsters (Padma *et al*., 1989a).

3.1.6 *Subcutaneous administration*

(*a*) *Rat*

Groups of male and female Fischer 344 rats, 9 weeks of age, received thrice weekly subcutaneous injections of NNK in trioctanoin for 20 weeks to give total doses of 0 (26 males and 26 females), 1.0 (27 males and 27 females), 3.0 (15 males and 15 females) or 9.0 (15 males and 15 females) mmol/kg bw. After 7 weeks, injections were interrupted for 2 weeks because of weight loss in the high-dose group. Animals were killed when moribund or when only 20% of the group were alive. Major organs were fixed and examined microscopically. The high dose of NNK resulted in the deaths of all rats by 60–70 weeks; the animals in the mid-dose group survived to approximately 110 weeks. Survival in low-dose NNK-treated rats was comparable with that of trioctanoin controls. Trioctanoin control rats did not develop lung tumours except for one adenoma in a female rat. Lung tumour incidence in male rats given 0, 1.0, 3.0 and 9.0 mmol/kg bw was 0/26, 23/27 ($p < 0.01$), 13/15 ($p < 0.01$) and 14/15 ($p < 0.01$), respectively; that in female rats was 1/26, 8/27 ($p < 0.05$), 7/15 ($p < 0.01$) and 8/15 ($p < 0.01$), respectively. Lung tumours were adenomas or adenocarcinomas; five male rats had also squamous-cell carcinomas. Nasal tumour inci-

dence in male control, low-, mid- and high-dose rats was 0/26, 20/27 (19 benign, one malignant; $p < 0.01$), 13/15 (six benign, seven malignant; $p < 0.01$) and 14/15 (four benign, 10 malignant; $p < 0.01$), respectively; that in female rats was 0/26, 10/27 (10 benign; $p < 0.01$), 12/15 (9 benign, three malignant; $p < 0.01$) and 14/15 (four benign and 10 malignant; $p < 0.01$), respectively. Liver tumour incidence in the four groups of male rats was 3/26 (three benign), 3/27 (two benign, one malignant), 4/15 (one benign, three malignant) and 6/15 (two benign, four malignant; $p < 0.05$), respectively; and that in female rats was 1/26 (one benign), 4/27 (three benign, one malignant), 4/15 (two benign, two malignant) and 5/15 (two benign, three malignant; $p < 0.05$), respectively (Hoffmann *et al*., 1984).

Groups of male Fischer 344 rats, 8 weeks of age, received thrice-weekly subcutaneous injections of 0.0055 mmol/kg bw NNK in trioctanoin or trioctanoin alone (control) for 20 weeks. Surviving animals were killed after 104 weeks and major organs were examined for the presence of tumours. Of rats injected with NNK, 13/27 had lung tumours, four of which were adenocarcinomas and nine were adenomas, versus 1/26 (adenoma) trioctanoin controls; 6/27 had nasal tumours, one of which was a squamous-cell carcinoma and five of which were squamous-cell papillomas, and 10/27 had liver tumours, two of which were hepatocellular carcinomas and eight of which were adenomas. None of the 26 trioctanoin control rats developed nasal or liver tumours (Hecht *et al*., 1986a).

Groups of male Fischer 344 rats [initial number and age unspecified] (weighing 175–200 g) received thrice-weekly subcutaneous injections of 0, 0.03, 0.1, 0.3, 1.0, 10 or 50 mg/kg bw NNK in trioctanoin for 20 weeks. Animals were killed over a 100-week period and were examined for tumours. Rats were also killed when they had a weight loss of 10–15% over a 2-week period compared with controls. Tumour incidence increased in a dose–responsive manner from 0.03 mg/kg to 50 mg/kg. At the highest dose of NNK, the incidence of lung hyperplasia was 93.5% (58/62) and that of benign and malignant lung tumours was 87.1% (54/62). The incidence of lung tumours increased with increasing dose: 2.5% of 40 rats at 0 mg/kg, 6.7% of 60 rats at 0.03 mg/kg, 10.0% of 60 rats at 0.1 mg/kg, 13.3% of 60 rats at 0.3 mg/kg, 53.3% of 30 rats at 1.0 mg/kg, 73.3% of 30 rats at 10 mg/kg and 87.1% of rats at 50 mg/kg. The majority of benign lung tumours were classified as solid adenomas (72 tumours), papillary adenomas (seven tumours) or mixed (five tumours). Among the malignant tumours, 11 were solid carcinomas, 46 were papillary carcinomas, three were mixed carcinomas and 16 were squamous-cell carcinomas. In the highest-dose group, a 34% (21/62) incidence of tumours was found in the respiratory region and a 50% (31/62) incidence of tumours was observed in the olfactory region. In the respiratory region, 18 of the tumours were benign and three were malignant. At 10 mg/kg, tumour incidence was 53% (16/30) in the respiratory region and 26% (8/30) in the olfactory region. The types of tumours found were: 15 benign and one malignant in the respiratory region and four benign and four malignant in the olfactory region. The remainder of the lower doses did not induce tumours in the nasal cavity (Belinsky *et al*., 1990).

(*b*) *Hamster*

Groups of 15 male and 15 female Syrian golden hamsters, 8–10 weeks of age, received thrice-weekly subcutaneous injections of 0 (control) or 10 mg NNK (99% pure) in 0.3 mL trioctanoin (total dose, 0.91 mmol per hamster). In a second experiment, groups of 10 male and 10 female hamsters received thrice-weekly subcutaneous injections of 2.5 mg NNK in 0.3 mL (total of 75 injections; total dose, 0.91 mmol per hamster). In the first experiment, only 50% of the hamsters survived after 10 weeks; by 14 weeks, survival was only 26%. In the second experiment, all animals were alive after 4 months, 80% after 7 months, 75% after 10 months and 30% after 13 months. The first experiment was terminated after 16 months and the second experiment after 17 months. In the first experiment, 8/15 males and 11/15 females had lung tumours. In the second experiment, 10/10 males and 6/10 females developed lung tumours. In the first experiment most of the tumours were adenomas; in the second experiment, 6/10 tumours in males were adenocarcinomas and 4/6 tumours in females were adenocarcinomas. No lung tumours were observed in the control hamsters (Hoffmann *et al*., 1981).

In a study that examined the effect of smoke inhalation on lung tumour formation, groups of 10 male and 10 female Syrian golden hamsters, 8 weeks of age, received a single subcutaneous injection of 0 (vehicle control), 1.0, 3.3 or 10 mg NNK in 0.3 mL trioctanoin. The experiment was terminated after 72 weeks. Statistical significance was determined using the χ^2 test. NNK (10 mg) plus sham smoking produced three lung adenomas in males and one lung adenoma in a female. At the mid-dose, NNK induced two lung adenomas in males and none in females; the low dose of NNK induced two lung adenomas in males and two in females; trioctanoin alone induced no tumours. When NNK was followed twice daily by an exposure period to tobacco smoke for 69 weeks, the incidence of lung tumours in females treated with 3.3 mg rose from 0/10 to 6/10 ($p < 0.01$) (Hecht *et al*., 1983a).

In a study to evaluate the effect of hyperoxia on lung tumour development produced by NNK, four groups of 15 male Syrian golden hamsters each received twice-weekly subcutaneous injections of 0 (two control groups) or 1.25 mg/kg bw NNK (two experimental groups) in 0.15 mL trioctanoin, and were maintained under either ambient air conditions or in hyperoxia chambers (oxygen concentration, 70%) for periods of 8–12 months (ambient air) or 12–16 weeks (70% oxygen). Animals were killed at intervals of 4 weeks and three hamsters per time-point were evaluated for tumour formation. The cumulative incidence of lung tumours (adenomas and mixed adenosquamous carcinomas) in animals treated with NNK alone was 80% (12/15). When animals were maintained in an atmosphere of 70% oxygen, 70% (10/14) of animals developed neuroendocrine or mixed neuroendocrine and squamous-cell tumours. Under hyperoxia, the latent period was reduced from 16 weeks to 8 weeks. No tumours were observed in control animals (Schuller *et al*., 1990).

(*c*) *Mink*

Groups of random-bred mink (originated from the breeding farm of the Norwegian College of Veterinary Medicine), 3 months of age, received twice-weekly subcutaneous injections of NNK (purity, > 99%) for 28 weeks (four females; total dose, 6.3 mM) or NNN + NNK (two males and four females; total doses, 11.9 mM + 6.3 mM). Survival ranged from 56 to 136 weeks for mink injected with NNK and from 16 to 130 weeks for mink injected with NNN + NNK. Control animals were killed at 156 weeks (one male and four females). NNK alone induced malignant tumours in the nasal cavity (mainly esthesioneuroepithelioma) with invasion into the forebrain in all four females; one of them also developed multiple lung tumours (adenomas and/or adenocarcinomas). Time to tumour was 77 ± 39 weeks. Following the combined treatment with NNK + NNN, all males developed tumours in the nasal cavity (esthesioneuroepithelioma) and invasion into the forebrain was observed at 39 and 40 weeks. Nasal cavity tumours (mainly esthesioneuroepithelioma) were induced in three females with invasion into the forebrain. Of the four females, one developed multiple lung tumours (adenomas and adenocarcinomas) and one a liver tumour (bile duct adenoma). Time to tumour was 58 ± 44 weeks. No tumours were observed in the control minks (Koppang *et al*., 1997).

3.1.7 *Transplacental or neonatal exposure*

(*a*) *Mouse*

Groups of male and female neonatal Cr:NIH (S) mice from 15 litters [initial number unspecified] received intraperitoneal injections of 50 mg/kg bw NNK in saline on days 1, 3, 5, 7 and 10. Controls consisted of eight litters that were injected with saline alone. Mice were killed at 15 months or when they showed signs of illness. Representative tumours were stained and classified as adenomas or carcinomas following microscopic examination. Statistical analysis was conducted using the Fisher's exact test for tumour incidence and Student's *t*-test for number of tumours per mouse. In male mice, 30/55 animals developed liver tumours (including four carcinomas) with an average of 1.15 ± 1.4 tumours per mouse, whereas no liver tumours were seen in the 33 control males. In females, 8/57 mice developed liver tumours (including two carcinomas) with a multiplicity of 0.14 ± 0.35 tumours per mouse and no liver tumours were observed in control females. Lung tumours [no histopathological details provided] were found in 56.6% of treated males (30/55) ($p < 10^{-7}$) with a multiplicity of 0.74 ± 0.9 versus 0.3 ± 0.6 tumours per mouse in 21% (7/33) of saline controls ($p < 0.025$, *t*-test). In females, lung tumours [no histopathological details provided] were observed in 36.8% (21/57) of NNK-treated mice versus 22% (7/32) of saline controls. The average number of tumours per lung in treated females was 0.51 ± 0.75 versus 0.25 ± 0.5 in saline controls ($p < 0.1$, *t*-test) (Anderson *et al*., 1991).

Pregnant Swiss (Cr:NIH) mice were treated either with 0 (untreated mothers) or with a close to maximum tolerated dose of 100 mg/kg bw NNK by intraperitoneal injection in

saline on gestation days 15, 17 and 19. Infant mice of untreated mothers received an intraperitoneal injection of either 50 mg/kg bw NNK in saline or saline alone on day 4. The number of animals in each group ranged from 27 to 30 and comprised male and female progeny from at least 10 litters. All animals were killed at 52 weeks. Statistical significance of tumour multiplicities was performed using the Kruskal-Walls ranking procedure for differences among treatment groups for organ/sex combinations and Wilcoxon rank-sum to make pairwise comparisons between treatment groups. NNK did not induce tumours transplacentally in male or female offspring. Infant male mice treated with NNK on postnatal day 4 developed both lung (incidence, 8/30 versus 2/27 controls; multiplicity, 0.27 ± 0.45 versus 0.07 ± 0.27 in controls) and liver tumours (mainly adenomas) (incidence, 7/30 versus 1/27 controls; multiplicity, 0.23 ± 0.50 versus 0.03 ± 0.18 in controls, $p = 0.035$). In postnatally treated females, the incidence of lung tumours was not significantly increased and no liver tumours occurred (Beebe *et al*., 1993). [The Working Group noted that the control group for transplacental treatment was used to perform statistics for the postnatal experiment.]

(*b*) *Hamster*

Groups of five pregnant Syrian golden hamsters received either a single subcutaneous injection of 50, 100 or 200 mg/kg bw NNK (> 98% pure) in trioctanoin on day 15 of gestation or multiple subcutaneous injections of 50 or 100 mg/kg bw NNK in trioctanoin on days 13, 14 and 15 of gestation. Tumour incidence was analysed by the paired *t*-test. No tumours were observed in 82 and 83 offspring of animals treated with a single or multiple injections of trioctanoin alone. After single injections of 50, 100 or 200 mg/kg bw NNK, tumours (all sites combined) were observed in 29% (11/38), 56% (20/36) and 76% (19/25) of male and 51% (17/35), 56% (20/36) and 61% (25/41) of female offspring ($p < 0.01$ versus controls for all six groups). After multiple injections of 50 and 100 mg/kg bw NNK, tumours (all sites combined) were observed in 49% (19/39) and 73% (29/40) and 63% (25/40) and 62% (23/37) of male and female offspring, respectively ($p < 0.01$ versus controls for all 4 groups). The incidence of respiratory tract tumours (nasal cavity, larynx, trachea) in offspring that received single injections of 50 mg/kg bw NNK was 21% (8/38) in males and 26% (9/35) in females; in those treated with 200 mg/kg bw, the incidence was 24% (6/25) in males and 61% (25/41) in females ($p < 0.05$ versus controls for all four groups). After multiple injections of NNK, the frequency of respiratory tract tumours in offspring treated with 50 mg/kg bw was 19% (7/37) in males and 33% (12/36) in females; in those treated with 100 mg/kg bw, the incidence was 38% (15/39) in males and 24% (9/38) in females ($p < 0.05$ versus controls for all four groups) (Correa *et al*., 1990).

Groups of four pregnant female Sendai virus-free Syrian golden hamsters were given 0 or 10% ethanol in the drinking-water from day 5 to day 16 of gestation and received a single intratracheal instillation of 50 mg/kg bw NNK (> 98% pure) in distilled water on day 15 of gestation. Controls were treated with either water alone or ethanol alone. No tumours were observed in the offspring of females treated with distilled water alone (0/28). Tumours developed in two offspring of mothers treated with ethanol alone (1/17 males, pancreas; and 1/23

females, lymphoma). Two adenocarcinomas of the olfactory region and one adrenal pheochromocytoma developed in 3/9 (33.3%) male offspring transplacentally exposed to NNK alone. In six female offspring, five adrenal pheochromocytomas, two colonic polyps, one liver tumour and three lymphomas were observed. In the offspring of mothers exposed to ethanol followed by NNK, tumours developed in 8/16 males and 13/17 females; adenocarcinomas of the nasal cavity were found in two males and two females ($p < 0.01$) [the Working Group calculated that this was not significant; $p = 0.11$, Fisher's exact test], ductular adenocarcinomas in the pancreas were observed in four males and 10 females ($p < 0.01$ compared with NNK alone), pheochromocytomas in the adrenals developed in three males and seven females ($p < 0.01$ compared with NNK alone) and one tumour in the colon occurred in one male and one female. No lymphomas were observed in this group (Schüller *et al.*, 1993).

Groups of pregnant Syrian golden hamsters received a single subcutaneous injection of 1, 5, 10 or 20 mg/kg bw NNK (> 98% pure) in trioctanoin on day 15 of gestation. Other groups of pregnant hamsters were given 0.05, 5 or 50 mg/kg bw NNK in distilled water by intratracheal instillation on day 15 of gestation. All control animals were given trioctanoin (15 males, 21 females) or distilled water alone (12 males, 15 females) and were killed at 59 weeks when the last NNK-treated hamsters were killed. Statistical analysis was performed by the paired *t*-test. Tumours were observed in NNK-exposed offspring at multiple sites including nasal cavity, adrenal glands, colon, pancreas and lymphoma. Total tumour incidence in the male and female offspring of mothers that received a subcutaneous injection was: 1 mg/kg bw, 27.3% in males (3/11) and 16.7% in females (3/18), 5 mg/kg bw, 27.3% in males (3/11) and 21.4% in females 3/14); 10 mg/kg bw, 33.3% in males (4/12) and 28.6% in females (2/7); and 20 mg/kg bw, 50% in males (3/6) and 57.2% in females (8/14). No tumours were observed in the trioctanoin controls. In offspring of mothers treated by intratracheal instillation, tumour incidence was: 0.05 mg/kg bw, 33.3% in males (2/6) and 50% in females (10/20); 5 mg/kg bw, 28.6% in males (4/14) and 42.1% in females (8/19); and 50 mg/kg bw, 33.3% in males (3/9) and 40% in females (6/15). No tumour occurred in distilled water controls. Tumours in NNK-exposed offspring were predominantly found in the nasal cavity and adrenal glands. The total tumour incidence in all NNK-exposed offspring was significantly increased ($p < 0.01$) with no significant difference between the routes of administration (Schüller *et al.*, 1994).

Groups of outbred female Syrian golden hamsters, 8 weeks of age, were given ethanol (10% v/v) in the drinking-water from day 5 through to day 15 of pregnancy. Some females were also instilled intratracheally with 50 mg/kg NNK on day 15 of pregnancy. Offspring were born on the evening of day 16 and were observed until clinical symptoms of pancreatic disease occurred. Groups of offspring were given either the cyclooxygenase inhibitor ibuprofen (infant Motrin oral suspension diluted with sterile water to yield 2.86 mg/kg given orally three times a week for life) or the 5-lipoxygenase-activating protein inhibitor MK886 (10 mg/kg dissolved in 0.25% carboxymethylcellulose in sterile water given orally thrice-weekly for life). Ten of 16 (62%) offspring of the hamsters given ethanol and NNK alone developed pancreatic ductal adenocarcinoma compared with 5% of controls. Significant

reductions (p = 0.0026) were observed in ibuprofen- (6/24) and MK886- (8/19) treated offspring (Schuller *et al.*, 2002).

3.1.8 *Administration with known carcinogens or modifying factors*

These studies have been reviewed comprehensively (Hecht, 1998). One study in mice and two studies in rats that were not included in this review are summarized below.

(*a*) *Mouse*

The NNK/mouse lung model has been used extensively by numerous investigators to determine factors, conditions, drugs or chemopreventive compounds that can modulate the formation of lung tumours in mice. One of these studies is summarized below.

A study was conducted to determine the capacity of cigarette smoke to induce lung tumours and promote lung tumorigenesis induced by NNK. Groups of 20 female A/J mice, 7 weeks of age, were exposed for 6 h per day on 5 days per week for 26 weeks to filtered air (FA), cigarette smoke (CS; diluted mainstream smoke (target concentration, 250 mg total particulate matter/m^3) from IR3 research cigarettes), NNK or NNK plus CS. Mice were exposed for 3 days to 50% of the target concentration of CS and for 4 days to 75% of the target concentration of CS before full exposure. Three days before CS exposure, mice received an intraperitoneal injection of 100 mg/kg bw NNK in 0.1 mL saline. Mice were killed 5 weeks after the exposures were terminated. Total tumours were enumerated macroscopically and characterized microscopically. Differences in survival were analysed by Breslow statistics in a Kaplan-Meier survival analysis. Student's *t*-test with a Bonferroni multiple comparisons correction was used to examine group differences in lung weight, tumour multiplicity for all animals and tumour multiplicity in tumour-bearing animals, with significance set at the $p < 0.05$ level. The lung tumour incidence among the four groups was: FA, 5/19 (26%); CS, 0/19 (0%); FA + NNK, 19/20 (95%); and CS + NNK, 13/16 (81%). The lung tumour multiplicities (total tumours/animal at risk) were: FA, 0.32 ± 0.58 tumours per animal; FA + NNK, 2.50 ± 1.67 tumours per animal; and CS + NNK, 2.50 ± 1.97. Those among tumour-bearing animals were: FA, 1.20 ± 0.44 tumours per animal; FA + NNK, 2.63 ± 1.61 tumours per animal; and CS + NNK, 3.08 ± 1.71. CS exposure decreased both body weights and lung weights, but treatment with NNK had no additional effect. Tumour multiplicity was greater in the FA + NNK- and the CS + NNK-treated groups compared with the FA- and CS-treated groups ($p < 0.05$) among all animals, but tumour multiplicity in the tumour-bearing animals did not differ between the FA-, FA + NNK- or CS + NNK-treated groups (Finch *et al.*, 1996). [The Working Group noted that animals were held for a relatively short period of exposure to let tumours develop].

(*b*) *Rat*

Groups of 30 male Fischer 344 rats, 10 weeks of age, were treated with a mixture of 0.5 mL NNK + NNN (total dose, 14 + 68 μg) dissolved in water by swabbing the oral cavity and lips of the animals with a cotton swab dipped into the solution. A group of 21

rats was used as water controls. Application was performed as follows: once a day for 7 days, twice a day for 5 days per week, once a day for 2 days in weeks 2–23 and twice a day from week 24 to 131. The mean approximate total dose of NNK was 19 mg per rat and that of NNN was 97 mg. The experiment was terminated at 131 weeks at which time survival was 14%. The incidence of oral tumours was 8/30 (six cheek papillomas, one hard palate papilloma and two tongue papillomas) in NNK + NNN-treated animals and 0/21 in controls ($p < 0.05$). In addition to oral tumours, four lung adenocarcinomas and one lung adenoma were found in five treated animals, and one lung adenoma developed in one control animal. The incidence of tumours in the prostate and mammary glands and that of leukaemia/lymphoma in treated animals did not differ from that in controls (Hecht *et al*., 1986b).

Groups of male Fischer 344 rats were maintained on a high-fat (HF) diet (23.5% corn oil) or on a low-fat (LF) diet (5.0% corn oil). NNK was administered in the drinking-water at a concentration of 0 or 2.0 ppm [mg/L]. The number of animals in the treatment groups was: NNK–HF, 60 rats; NNK–LF, 60 rats; tap-water–HF, 20 rats; tap-water–LF, 20 rats. The experiment was terminated after 95–105 weeks. Incidences of lung tumours at termination were: NNK–HF, 30/60; NNK–LF, 27/60; HF, 1/20; LF, 1/20. Lung tumours were mainly adenomas or adenocarcinomas. There was no significant difference in the final incidence of lung tumours between NNK–HF and NNK–LF groups, but significantly survival was shorter in the NNK–HF than in the NNK–LF group. The incidence of pancreatic tumours was: NNK–HF, 28/60 ($p < 0.05$); NNK–LF, 19/60; HF, 6/20; LF, 6/20. In the NNK–HF group, 18 rats had benign and malignant tumours of the exocrine pancreas; in addition, 10 islet-cell tumours were observed. In the NNK–LF group, the corresponding numbers were 14 and five (Hoffmann *et al*., 1993a).

3.1.9 *Carcinogenicity of NNK metabolites*

Mouse

Groups of 25 female strain A/J mice received thrice weekly intraperitoneal injections of 0.01 mL of a 1% solution of NNK-*N*-oxide suspended in trioctanoin for 7.3 weeks (22 injections; total dose, 0.11 mmol/mouse) and were held for 30 weeks after the last injection. Twenty-four of 25 mice treated with NNK-*N*-oxide developed lung tumours (24 carcinomas/90 tumours) at a multiplicity of 3.6 ± 2.7 tumours per mouse. One NNK-*N*-oxide-treated mouse had a leiomyoma of the uterus. In untreated animals, 10/25 animals developed lung tumours with a multiplicity of 0.6 ± 0.9 tumours per surviving mouse whereas 4/25 trioctanoin controls developed lung tumours with a multiplicity of 0.2 ± 0.5 tumours per mouse (Castonguay *et al*., 1983b).

3.2 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL)

The carcinogenicity of NNAL in experimental animals has been evaluated previously (IARC, 1985).

3.2.1 *Intraperitoneal administration*

Mouse

A group of 25 female strain A/J mice, 6–8 weeks of age, received thrice-weekly intraperitoneal injections of 0.2 mL of a 0.5% solution of NNAL (purity > 99%) in saline for 7 weeks (total of 22 injections; total dose, 22 mg [0.11 mmol]) and were held without further treatment for an additional 30 weeks. Further groups of 25 female mice served as untreated and vehicle controls. Histological examination of lung and other organs that showed macroscopic lesions revealed lung adenomas in 1/25 untreated controls, 3/25 vehicle controls and 9/25 NNAL-treated mice (vehicle controls compared with treated mice, $p = 0.047$). No malignant tumours were observed in the lung or at other sites in any of the groups (Hecht *et al*., 1978).

Groups of 25 female strain A/J mice, 6–8 weeks of age, received thrice-weekly intraperitoneal injections of a 1% NNAL (> 99% pure) suspension in 0.1 mL trioctanoin for 7.3 weeks (22 injections; total dose, 0.11 mmol/mouse). Treated mice were held for 30 weeks after the last injection. Twenty-five untreated and 15 vehicle-treated female mice served as controls. Lung tumours were examined macroscopically for total lesions and microscopically for histological evaluation. The Student's *t*-test was used to determine statistical significance. Of the NNAL-treated mice, 25/25 developed 658 lung tumours (243 carcinomas) at a multiplicity of 26.3 ± 11.7 tumours per surviving mouse ($p < 0.0001$ compared with vehicle controls). Two NNAL-treated mice developed extrapulmonary tumours: a squamous-cell papilloma of the nasal cavity and a papilloma of the tongue. In untreated animals, 10/25 developed tumours with a multiplicity of 0.6 ± 0.9 tumours per surviving mouse. In trioctanoin controls, 4/25 animals had tumours with a multiplicity of 0.2 ± 0.5 tumours per surviving mouse (Castonguay *et al*., 1983a).

Two groups of 30 and 20 female strain A/J mice, 6–8 weeks of age, received thrice-weekly intraperitoneal injections of a total dose of 10 μmol and 50 μmol NNAL in 0.1 mL saline for 7 weeks. After 30 weeks, animals were killed and lung adenomas were enumerated. Statistical analysis was conducted by analysis of variance (nested model) followed by Scheffe's test for multiple comparison of means. Controls were 30 mice injected with saline alone (negative controls), 30 mice injected with 5 μmol NNK and 20 mice injected with 20 μmol NNK (positive controls). In NNAL-treated mice, 22/30 low-dose females had tumours with a tumour multiplicity of 1.5 ± 1.4 tumours per mouse and 20/20 high-dose females had tumours with a multiplicity of 9.7 ± 6.4 tumours per mouse. In the saline controls, 8/30 animals had tumours with a tumour multiplicity of 0.27 ± 0.58 tumours per mouse. In the NNK controls, the tumour incidence was 23/30 in low-dose females with a tumour multiplicity of 1.6 ± 1.2 tumours per mouse and 20/20 in high-dose females with a tumour multiplicity of 9.2 ± 6.3 tumours per mouse (Amin *et al*., 1996).

Groups of 14–20 female strain A/J mice, 7 weeks of age, received a single intraperitoneal injection of 20 μmol NNAL or a metabolite of NNAL [4-(methylnitrosamino)-1-(3-pyridyl)but-(*S*)-1-yl] β-*O*-D-glucosiduronic acid, 4-(methylnitrosamino)-1-(3-pyridyl-*N*-oxide)-1-butanol, 5-(3-pyridyl)-2-hydroxytetrahydrofuran, 4-(3-pyridyl)butane-1,4-diol or

2-(3-pyridyl)tetrahydrofuran) in 0.2 mL of saline. A control group of 20 female mice was injected with saline only. Mice were killed after 16 weeks and lung tumours enumerated. Statistical analysis was carried out using ANOVA and χ^2. In saline controls, 2/20 animals developed lung tumours (adenomas) with a tumour multiplicity of 0.1 ± 0.3 tumours per mouse. None of the five NNAL metabolites was tumorigenic in the mouse lung; 20/20 NNAL-treated mice developed lung adenomas ($p < 0.001$) with a tumour multiplicity of 12.1 ± 5.6 tumours per mouse ($p < 0.0001$) (Upadhyaya *et al.*, 1999).

3.2.2 *Administration in the drinking-water*

Rat

A group of 30 male Fischer 344 rats, 8 weeks of age, was given 5.0 ppm NNAL (> 99% pure) in the drinking-water throughout the experimental period. Animals were killed at 112 weeks. A group of 80 male rats (drinking-water only) served as controls. Lung tumours developed in 26/30 NNAL-treated rats; five rats had adenomas, 12 rats had adenocarcinomas ($p < 0.01$) and nine rats had adenosquamous carcinomas ($p < 0.01$). Lung tumours developed in 6/80 drinking-water controls: three adenomas, two adenocarcinomas and one adenosquamous carcinoma. The NNAL-treated rats also developed pancreatic tumours (8/30; $p < 0.01$). Three rats had acinar adenomas, four had ductal adenocarcinomas and one had an acinar adenocarcinoma. One control rat had an acinar adenoma (Rivenson *et al.*, 1988).

3.2.3 *Administration with known carcinogens or modifying factors*

(*a*) *Mouse*

Female Hid:SENCAR BR mice, 50–55 days of age, received topical applications of 2.8 μmol/mouse NNAL in 100 μL acetone every other day (10 doses; total dose, 28 μmol/mouse). Twice weekly applications of 2.0 μg TPA began 10 days after the last NNAL treatment for 20 weeks. Control mice were treated with acetone followed by TPA. Statistical analysis was by χ^2 test. All mice were examined macroscopically for skin tumours and for tumours in lung and liver. Skin tumours developed in 2/29 mice given NNAL. No tumours were observed in the 29 controls and none of the NNAL-treated animals developed lung or liver tumours (LaVoie *et al.*, 1987).

(*b*) *Hamster*

The effects of administration of low doses of NNAL were investigated in Syrian golden hamsters treated with *N*-nitrosobis(2-oxopropyl)amine (BOP). Three groups of 30 female Syrian golden hamsters, 5 weeks of age, were given a single subcutaneous injection of 10 mg/kg bw BOP. After this treatment, animals were given drinking-water alone, or drinking-water supplemented with 2 ppm or 5 ppm NNAL during weeks 2–53. Three additional groups of 10, 20 and 20 hamsters were given tap-water alone, 2 ppm NNAL or 5 ppm NNAL, respectively. NNAL did not influence the incidence of pancreatic adenocarcinomas

or dysplastic lesions. However, the total incidence of pancreatic adenocarcinomas and dysplastic lesions was significantly higher ($p < 0.05$) in the BOP/high-dose NNAL group (14/30) than in the groups treated with BOP alone (5/27) or BOP/low-dose NNAL (4/29). NNAL itself did not induce any proliferative lesions of the exocrine pancreas. No effects were found on the incidence or multiplicity of pancreatic islet-cell proliferative lesions. (Furukawa *et al*., 1997).

3.3 *N′*-Nitrosonornicotine (NNN) (Table 10)

The carcinogenicity of NNN in experimental animals has been evaluated previously (IARC, 1985).

3.3.1 *Intraperitoneal administration*

(*a*) *Mouse*

A group of 20 male and 20 female Chester Beatty mice, 6 weeks of age, received weekly intraperitoneal injections of 0.1 mL 2% NNN dissolved in arachis oil for 41 weeks. Controls were 15 male and 15 female mice that received arachis oil only. During the first 7 months of NNK treatment, 14 males and 11 females died with no evidence of tumours. However, after the 8th month, eight animals died. Seven of these (five females and two males) were autopsied and were found to have multiple pulmonary adenomas that were confirmed histologically. In five animals, the number of pulmonary lesions was greater than 30. In addition, one of the males had a lymphosarcoma in the kidney (Boyland *et al*., 1964a).

Groups of 25 strain A/J female mice, 6–8 weeks of age, received a total of 22 intraperitoneal injections of 0.5% NNN in 0.2 mL saline or 1% NNN in 0.1 mL trioctanoin over a period of 7 weeks (total dose, 22 mg per mouse). After the final injection, mice were held for an additional 30 weeks. Controls comprised untreated animals and vehicle controls (saline and trioctanoin). Statistical comparisons were made using the Student's *t*-test. Animals injected with NNN in saline had lung tumour incidence of 76% (16/21) with a tumour multiplicity of 1.74 ± 1.37 tumours per mouse compared with a tumour incidence of 12% (3/25) and a multiplicity of 0.24 ± 0.72 tumours per mouse in the saline controls. Animals injected with NNN in trioctanoin had a lung tumour incidence of 57% (12/23) and a tumour multiplicity of 0.87 ± 1.01 tumours per mouse compared with a 21% (5/24) incidence and 0.2 ± 0.41 tumours per mouse multiplicity in vehicle controls. NNN induced significant increases in tumour incidence over vehicle controls ($p < 0.05$) (Hecht *et al*., 1978).

Groups of 25 female strain A/J mice, 6–8 weeks of age, received thrice-weekly intraperitoneal injections for 7.3 weeks (22 injections) of NNN (> 99% pure) suspended in 0.1 mL saline (total dose, 0.12 mmol/mouse). Mice were held for 30 weeks after the last injection. Untreated and vehicle-treated animals served as controls. Lung tumours were counted macroscopically when animals were killed and fixed in 10% formalin for histo-

logical evaluation. The Student's *t*-test was used to determine statistical significance. In NNN-treated animals, 16/24 had lung tumours with a multiplicity of 1.2 ± 1.3 tumours per surviving mouse ($p < 0.05$ compared with vehicle controls). In untreated animals, 10/25 developed lung tumours with a multiplicity of 0.6 ± 0.9 tumours per surviving mouse; in saline controls, 7/24 animals had lung tumours with a multiplicity of 0.4 ± 0.6 tumours per surviving mouse. Most of the tumours were classified as lung adenomas, except for 10 carcinomas in NNN-treated animals (Castonguay *et al*., 1983a).

Groups of 30 female strain A/J mice, 6–8 weeks of age, received thrice-weekly intraperitoneal injections of NNN (considered pure by HPLC and TLC) in 0.2 mL saline for 7 weeks (total dose, 100 μmol/mouse). Mice were held for 30 weeks after the last injection. The Student's *t*-test was used to determine statistical significance. NNN induced an 83% (25/30) ($p < 0.0001$) incidence of lung tumours with a multiplicity of 1.8 ± 1.4 tumours per mouse; the saline controls had a tumour incidence of 40% (12/30) with a tumour multiplicity of 0.5 ± 0.7 tumours per mouse (Hecht *et al*., 1988a).

Two groups of 30 female strain A/J mice, 6–8 weeks of age, received thrice-weekly intraperitoneal injections of a total dose of 40 μmol or 200 μmol NNN in 0.1 mL saline for 7 weeks. After 30 weeks, animals were killed and lung adenomas were enumerated. Statistical analysis was conducted by analysis of variance (nested model) followed by Scheffe's test for multiple comparison of means. Controls were 30 mice injected with saline alone (negative controls), 30 mice injected with 5 μmol NNK and 20 mice injected with 20 μmol NNK (positive controls). In NNN-treated mice, the incidence of tumours was 43.3% [13/30] and 80% [24/30] ($p < 0.001$); tumour multiplicity was 0.7 ± 1.0 and 2.3 ± 2.1 tumours per mouse in the low- and high-dose group, respectively. In the saline controls, tumour incidence was 26.7% [8/30] and tumour multiplicity was 0.27 ± 0.58 tumours per mouse. In the NNK controls, tumour incidence was 76.7% [23/30] and 100% [20/20] and tumour multiplicity was 1.6 ± 1.2 and 9.2 ± 6.3 tumours per mouse in animals treated with 5 and 20 μmol, respectively (Amin *et al*., 1996).

(*b*) *Hamster*

Male Syrian golden hamsters, 8 weeks of age, were placed on a liquid diet (#711, Bio-Serv, Frenchtown, NJ). At 9 weeks of age, animals were divided into two groups of 105 each; one of these groups was placed on a liquid diet that contained ethanol (6% w/v). At 13 weeks of age, each group was subdivided into five groups of 21 animals; two groups received thrice-weekly intraperitoneal injections of 1 mmol (2.37 mg) or 2 mmol (4.75 mg) NNN in saline for 25 weeks (total doses, 1 mmol and 2 mmol per hamster). After 16 weeks on the liquid diets, a significant decrease in weight was observed; all animals were placed on an NIH-07 diet for 1 month and injections were suspended. Liquid diets were reinstated and, after 1 week, injections were resumed without further interruption. Animals were killed and autopsied when moribund and all remaining animals were killed 15 months after the first injection. None of the 19 ethanol-treated or 21 diet control animals developed tumours in the nasal cavity or trachea. Approximately 50% of the control animals and NNN-treated animals developed adrenal tumours. Five of 21 NNN-

treated (1 mmol) animals had tumours (one nasal cavity and four tracheal; $p < 0.05$). When the diet was supplemented with ethanol, 6/17 NNN-treated (1 mmol) animals had tumours (one nasal cavity and five tracheal). After treatment with 2 mmol, 13/21 animals had tumours (five nasal cavity and nine tracheal) ($p < 0.001$); in the ethanol-supplemented group, 10/21 had tumours (four nasal cavity and seven tracheal) (McCoy *et al*., 1981).

3.3.2 *Skin application*

Mouse

NNN (purity, > 98.5%) was applied twice a week to the interscapular region of female CFLP mice at doses of 12.5, 50 and 200 µg/mouse in 200 µL acetone for 104 weeks. No skin tumours appeared in 65 acetone-treated controls. The 65 mice treated with 12.5 µg developed three skin carcinomas; in 65 mice treated with 50 µg, two skin papillomas and one skin carcinoma developed. In 64 mice treated with 200 µg, three skin carcinomas were observed (Deutsch-Wenzel *et al*., 1985).

3.3.3 *Oral administration*

(*a*) *Mouse*

Groups of male BALB/c mice, 8 weeks of age, were untreated or received 1 mg NNN in distilled water deposited on the tongue thrice weekly (total dose, 72 mg NNN) and groups of male Swiss mice, 8 weeks of age, were untreated or received 0 (vehicle control) or 1 mg NNN in distilled water deposited on the tongue thrice weekly (total doses, 22 or 72 mg NNN). Animals were killed at 22 months or when moribund. Tumours were observed in 13/19 (68%) Swiss mice treated with the low dose of NNN (eight lung adenomas and seven forestomach papillomas) and in 5/10 (50%) Swiss mice treated with the high dose of NNN (three lung adenomas, two forestomach papillomas and two hepatomas) and 6/6 (100%) NNN-treated BALB/c mice (four lung adenomas, two forestomach papillomas and two hepatomas). Tumours [not specified] developed in 2/18 (11%) untreated Swiss mice and no tumours were observed in 14 untreated BALB/c mice or 11 Swiss mice given distilled water only (Padma *et al*., 1989a).

(*b*) *Rat*

A group of 20 male Fischer 344 rats [age unspecified] was given 0.02% NNN in the drinking-water on 5 days per week and tap-water on weekends for a period of 30 weeks (total dose, 630 mg). Moribund animals were killed and autopsied and the remaining animals were killed after 11 months. A group of 19 control rats given drinking-water only did not develop tumours in any major organs. Rats treated with NNN developed oesophageal tumours (12/20; 11 papillomas and three carcinomas) [$p < 0.0001$, Fisher's exact test]. Three of 20 rats developed nasal cavity carcinomas and one rat had a pharyngeal papilloma (Hoffmann *et al*., 1975).

Groups of 12 male and 12 female Fischer 344 rats, 6–8 weeks of age, were given NNN in the drinking-water at a concentration of 0 (control) and 0.012% (total dose, 3.6 mmol for males and 3.3 mmol for females) for a period of 36 weeks after which time animals received tap-water. The experiment was terminated after 104 weeks. Statistical significance was analysed using the χ^2 test. In males, NNN induced a total of 12 papillomas and three squamous-cell carcinomas in the oesophagus in 12/12 animals. In females, NNN induced a total of 11 papillomas and three squamous-cell carcinomas in 11/12 animals. In the nasal cavity, NNN induced eight papillomas and six malignant tumours in 10/12 males and seven papillomas and nine malignant tumours in 11/12 females. No oesophageal or nasal tumours were observed in 12 male or 12 female controls. The incidence of Leydig-cell tumours in males or mammary tumours in females was not increased in treated rats (Hecht *et al*., 1983b).

Groups of male Fischer 344 rats, 9 weeks of age, were given an ethanol diet (Groups 2 and 6) or a control liquid diet (#711, Bioserv, Frenchtown, NJ; Groups 1 and 5). At 13 weeks of age, animals in Groups 1 and 2 (26 rats each) received thrice-weekly subcutaneous injections of 0.3–0.5 mL saline. Groups 5 and 6 (30 rats each) began a liquid diet that contained NNN (17.5 mg/L) and ethanol and NNN, respectively. After 27 weeks, Groups 5 and 6 were placed on standard diet until they were killed at 98 weeks of age. Animals treated with saline developed no nasal cavity tumours. In Groups 5 and 6, most of the tumours observed in the nasal cavity were benign (11 and 20 benign and seven and six malignant, respectively). Significant numbers of benign and malignant tumours (squamous-cell carcinomas) were found in the oesophagus (16 and 13 benign and nine and seven malignant, respectively) (Castonguay *et al*., 1984a).

(*c*) *Hamster*

Groups of 10 male and 10 female Syrian golden hamsters, 6–7 weeks of age, were given 0 (control) and 0.016% NNN (purity, > 98%) in the drinking-water for 31 weeks, after which animals received tap-water. Total doses of NNN were estimated to be 1.9 mmol for males and 2.8 mmol for females. The experiment was terminated after 96 weeks. Statistical significance was analysed using the χ^2 test. NNN induced two nasal cavity tumours and one tracheal tumour in both males and females (papillomas). A lymphoma in the caecum and a liver angiosarcoma were observed in NNN-treated males. No tracheal or nasal tumours were observed in 10 male or 10 female control hamsters, although one female developed a lymphoma (Hecht *et al*., 1983b).

3.3.4 *Cheek pouch application*

Hamster

A group of 36 male and female [assumed to be equally distributed] Syrian golden hamsters [age unspecified] had one cheek pouch painted with 10 mg NNN (98% pure) in mineral oil five times a week for 24 weeks; in 16 control male and female [assumed to be equally distributed] hamsters, one buccal pouch was treated with mineral oil alone. Each

dose of NNN was approximately 10 mg. After 24 weeks, none of the animals had developed tumours (Papageorge *et al.*, 1996).

3.3.5 *Subcutaneous administration*

(*a*) *Rat*

Groups of 26 and 30 male Fischer 344 rats, 9 weeks of age, were given an ethanol diet (Groups 2 and 6) or a liquid diet (#711, Bio-Serv, Frenchtown, NJ; Groups 1 and 3). At 13 weeks of age, animals in Groups 1 and 2 (26 rats each) received thrice-weekly subcutaneous injections of 0.3–0.5 mL saline. Groups 3 and 4 (30 rats each) received thrice-weekly subcutaneous injections of 10 mg/kg bw NNN in saline (56–66 injections; total dose, 1 mmol/rat, respectively). Animals were killed at 98 weeks of age. Animals treated with saline developed no nasal cavity tumours. Most of the tumours in the nasal cavity were malignant in Groups 3 (20/24) and 4 (20/22). Only two benign and one malignant tumours were found in the oesophagus in Group 4 (Castonguay *et al.*, 1984a).

Groups of male and female Fischer 344 rats, 9 weeks of age, received thrice-weekly subcutaneous injections of NNN in trioctanoin for 20 weeks (total doses, 1.0, 3.0 or 9.0 mmol/kg bw) and were killed when moribund or when only 20% of the animals were alive. A control group was injected with trioctanoin only. Major organs were fixed and examined microscopically. The high dose of NNN resulted in the deaths of all rats (15 males and 15 females) by 60–70 weeks; rats treated with the mid-dose (15 males, 15 females) survived to approximately 110 weeks. Survival of low-dose NNN-treated rats (27 males, 27 females) was comparable with that of trioctanoin controls. The group of 26 male and 26 female trioctanoin controls did not develop any tumours. The incidence of benign and malignant nasal cavity tumours in male and female rats was 56% (15/27) and 44% (12/27) in the low-dose group, 73% (11/15) and 60% (9/15) in the mid-dose group and 86% (12/14) and 100% (15/15) in the high-dose group, respectively. At the high dose, all of the nasal cavity tumours were malignant (Hoffmann *et al.*, 1984).

(*b*) *Hamster*

Groups of 10 female and 10 male Syrian golden hamsters, 8–10 weeks old, received thrice-weekly subcutaneous injections of 5 mg NNN in 0.5 mL saline for 25 weeks (total dose, 375 mg). Controls were treated with saline only. Moribund animals were killed and autopsied; the remaining animals were killed after 83 weeks. Papillary tumours of the trachea occurred in 7/9 treated females and 5/10 treated males. Nasal cavity tumours were found in 0/10 females and 1/10 males (one adenocarcinoma). No nasal or tracheal tumours developed in the controls (Hilfrich *et al.*, 1977).

Groups of 15 male and 15 female Syrian golden hamsters, 8–10 weeks of age, received thrice-weekly subcutaneous injections of 8.6 mg NNN (> 99% pure) in 0.3 mL trioctanoin, (total dose, 0.91 mmol/hamster). In a second experiment, groups of 10 male and 10 female hamsters received a total of 75 subcutaneous injections of 2.15 mg NNN in 0.3 mL trioctanoin (total dose, 0.91 mmol/hamster). In the first experiment, treatment with NNN

did not result in early mortality. In the second experiment, all animals were alive after 4 months, 90% after 7 months, 80% after 10 months and 60% after 13 months. The first experiment was terminated after 16 months and the second experiment after 17 months. In the first experiment, 3/15 males and 2/15 females developed tracheal tumours (papillomas) and 1/15 females developed an adenoma of the lung. In the second experiment, 0/10 males and 2/10 females developed respiratory tumours (one lung adenocarcinoma and one tracheal tumour) (Hoffmann *et al*., 1981).

(*c*) *Mink*

Thirteen female and seven male random-bred mink (originated from the breeding farm of the Norwegian College of Veterinary Medicine), 3 weeks of age, received twice-weekly subcutaneous injections of NNN in sterile water for 38 weeks. Injections from the beginning to week 7 contained 1.5 mg NNN/mink and the dose was increased to 30 mg at week 8. The total dose was 2130 mg for both males and females, although the dose per kilogram body weight was twofold for females. Animals were killed when moribund and autopsies were performed. Among females, 13/13 developed nasal tumours (mainly esthesioneuroepithelioma) that invaded into the forebrain. In males, 1/7 developed a nasal tumour that also invaded the forebrain and five developed localized nasal tumours. Time to tumour was 128 ± 23 weeks after the first NNN injections. No tumours were observed in four control female animals (Koppang *et al*., 1992).

Random-bred mink (originated from the breeding farm of the Norwegian College of Veterinary Medicine), 3 months of age, received twice-weekly subcutaneous injections of NNN (purity, > 99%) for 28 weeks (total dose, 11.9 mM). Two males and four females received injections of NNN + NNK (total doses, 11.9 mM + 6.3 mM). Survival ranged from 69 to 156 weeks for mink injected with NNN and from 16 to 130 weeks for mink inoculated with the combination. Control animals were killed at 156 weeks. In the two males, NNN alone induced two malignant tumours (esthesioneuroepitheliomas) in the nasal cavity, one of which invaded the forebrain. In three females, three nasal tumours (esthesioneuroepitheliomas) developed with two invading the forebrain. In both males that received NNN + NNK, tumours were induced in the nasal cavity (esthesioneuroepitheliomas) and invasion into the forebrain was observed at 39 and 40 weeks. In the females, nasal cavity tumours (esthesioneuroepitheliomas) were induced with invasion into the forebrain; in addition, multiple lung tumours (mainly esthesioneuroepitheliomas) developed in one female and a liver tumour (bile duct adenoma) in another. Time to tumour was 58 ± 44 weeks. No tumours were observed in the control minks (Koppang *et al*., 1997).

3.3.6 *Administration with known carcinogens or modifying factors*

(*a*) *Mouse*

Female Hcfd:SENCAR BR mice, 50–55 days of age, received topical applications of 2.8 μmol NNN in 100 μL acetone every other day (total dose, 28 μmol/mouse). Twice-weekly applications of 2.0 μg 12-*O*-tetradecanoylphorbol-13-acetate (TPA) began 10 days

after the last NNN treatment and were continued for 20 weeks. Controls consisted of mice treated with acetone followed by TPA . Significance was analysed using χ^2 test. All mice were examined macroscopically for skin tumours and for tumours in the lung and liver. Mice initiated with NNN developed 0.07 skin tumours per mouse (tumour incidence, 2/27), an incidence that was twofold lower than that in the acetone controls (0.14 skin tumour/mouse; tumour incidence, 4/28) (LaVoie *et al*., 1987).

(*b*) *Rat*

Groups of 30 male Fischer 344 rats, 10 weeks of age, were treated with a mixture of 0.5 mL NNK + NNN (total dose, 14 + 68 μg) dissolved in water by swabbing the oral cavity and lips of the animals with a cotton swab dipped into the solution. A group of 21 rats was used as water controls. Application was performed once a day for 7 days, twice a day for 5 days per week, once a day for 2 days in weeks 2–23 and twice a day from week 24 to week 131. The mean approximate total dose of NNK was 19 mg per rat and that of NNN was 97 mg. The experiment was terminated at 131 weeks at which time survival was 14%. The incidence of oral tumours was 8/30 (six cheek papillomas, one hard palate papilloma and two tongue papillomas) in NNK + NNN-treated animals and 0/21 in controls ($p < 0.05$). In addition to the oral tumours, four lung adenocarcinomas and one lung adenoma were found in five treated animals. One lung adenoma was found in one control animal. The incidence of tumours in the prostate and mammary glands and that of leukaemia/lymphoma did not differ between treated animals and controls (Hecht *et al*., 1986b).

3.3.7 *Carcinogenicity of NNN metabolites*

(*a*) *Intraperitoneal injection*

Mouse

Groups of 25 female strain A/J mice, 6–8 weeks of age, received thrice-weekly intraperitoneal injections for 7.3 weeks (22 injections) of 3′-hydroxy-NNN, 4′-hydroxy-NNN or NNN-1-*N*-oxide suspended in 0.1 mL saline (total dose, 0.12 mmol/mouse) Mice were held for 30 weeks after the last injection. Untreated and vehicle-treated animals served as controls. Lung tumours were counted macroscopically when animals were killed and fixed in 10% formalin for histological evaluation. The Student's *t*-test was used to determine statistical significance. In 3′-hydroxy-NNN-, 4′-hydroxy-NNN- and NNN-1-*N*-oxide-treated mice, tumour multiplicities were 0.9 ± 1.4, 1.6 ± 1.5 ($p < 0.05$) and 0.8 ± 0.7 tumours per surviving mouse, respectively. Tumour incidences were 12/25, 19/25 and 16/25, respectively. In untreated animals, 10/25 developed lung tumours with a multiplicity of 0.6 ± 0.9 tumours per surviving mouse; in saline controls, 7/24 animals had lung tumours with a multiplicity of 0.4 ± 0.6 tumours per surviving mouse (Castonguay *et al*., 1983a).

(*b*) *Administration in drinking-water*

(i) *Rat*

Groups of 12 male and 12 female Fischer 344 rats, 6–8 weeks of age, were given NNN-1-*N*-oxide in the drinking-water at a concentration of 0.012% for a period of 36 weeks, after which time animals received tap-water (total estimated doses, 3.9 mmol for males, 2.9 mmol for females). The experiment was terminated after 104 weeks. Statistical significance was analysed using the χ^2 test. In males, NNN-1-*N*-oxide induced a total of five papillomas and three squamous-cell carcinomas in the oesophagus in 7/12 animals. In females, NNN-1-*N*-oxide induced a total of three carcinomas in the oesophagus in 3/12 animals. In the nasal cavity, NNN-1-*N*-oxide induced a total of five papillomas and seven malignant tumours in 11/12 males and a total of three papillomas and four malignant tumours in 7/12 females. No oesophageal or nasal tumours were observed in 12 male or 12 female controls. The incidence of Leydig-cell tumours in males or mammary tumours in females was not increased in treated rats (Hecht *et al*., 1983b).

(ii) *Hamster*

Groups of 10 male and 10 female Syrian golden hamsters, 6–7 weeks of age, were given NNN-1-*N*-oxide (purity, > 98%) in the drinking-water for 31 weeks, after which animals received tap-water. Total doses were estimated as 2.1 mmol for males and 2.3 mmol for females. The experiment was terminated after 96 weeks. Statistical significance was analysed using the χ^2 test. NNN-1-*N*-oxide failed to induce either nasal cavity or tracheal tumours in males or females, although one caecum adenoma in males and two colon adenomas and five malignant tumours of different sites in females were observed in animals treated with NNN-1-*N*-oxide. No tracheal or nasal tumours were observed in 10 male or 10 female control hamsters, although one female developed a lymphoma (Hecht *et al*., 1983b).

3.4 *N′*-Nitrosoanabasine (NAB)

The carcinogenicity of NAB in experimental animals has been evaluated previously (IARC, 1985).

3.4.1 *Intraperitoneal administration*

Mouse

A group of 31 female strain A/J mice, 6–8 weeks of age, received thrice-weekly intraperitoneal injections of a total dose of 100 μmol NAB in 0.1 mL saline for 7 weeks. After 30 weeks, animals were killed and lung adenomas were enumerated. Statistical analysis was conducted by analysis of variance (nested model) followed by Scheffe's test for multiple comparison of means. Control mice were injected with saline alone (negative controls; 30 mice) or with 5 (30 mice) or 20 (20 mice) μmol NNK (positive controls). In NAB-treated mice, the incidence of tumours was 90.3% [28/31] and tumour multiplicity was 1.8 ± 1.1

tumours per mouse. In the negative controls, tumour incidence was 26.7% [8/30] with a tumour multiplicity of 0.27 ± 0.58 tumours per mouse. In the positive controls, the tumour incidence was 76.7% [23/30] with a tumour multiplicity of 1.6 ± 1.2 tumours per mouse (5 μmol NNK) and 100% [20/20] with a tumour multiplicity of 9.2 ± 6.3 tumours per mouse (20 μmol NNK) (Amin *et al*., 1996).

3.4.2 *Administration in the drinking-water*

Rat

Groups of 16 male and 16 female Chester Beatty strain albino rats, approximately 7 weeks of age, were given drinking-water that contained 0.2% NAB [purity not specified] *ad libitum* on 6 days per week [presumably continuously]. The estimated daily dose was 5 mg/day and animals were killed when moribund or sick, at various intervals between 251 and 550 days of study. A group of 16 males and 16 females served as untreated controls. All but two female rats in the treated group were subjected to post-mortem examination. Of the 16 treated males, four had oesophageal carcinomas, nine had oesophageal papillomas and three had no tumour; of the 14 treated females, one had an oesophageal carcinoma, 11 had oesophageal papillomas and two had no tumour. No oesophageal tumour was reported in the control rats (Boyland *et al.*, 1964b).

A group of 20 male Fischer 344 rats, 7 weeks of age, was given 0.02% NAB in the drinking-water for 5 days a week and tap-water on weekends for a period of 30 weeks (total dose, 630 mg). Moribund animals were killed and autopsied and the remaining animals were killed after 11 months. A group of 19 control rats that was given drinking-water only did not develop tumours in any major organ. Rats treated with NAB developed one oesophageal papilloma (1/17) and one pharyngeal papilloma (1/20). No tumours were observed in the nasal cavity (Hoffmann *et al*., 1975).

3.4.3 *Subcutaneous administration*

Hamster

Groups of 10 male and 10 female Syrian golden hamsters, 8–10 weeks of age, received thrice-weekly subcutaneous injections of 5 mg NAB in 0.5 mL saline for 25 weeks (total dose, 375 mg). Controls (10 males and 10 females) were treated with saline only. Moribund animals were killed and autopsied; the remaining animals were killed after 83 weeks. No tumours were found in the NAB-treated animals. In control rats, no nasal or tracheal tumours developed; one female had a fibrovascular polyp in the uterus (Hilfrich *et al*., 1977).

3.5 *N′*-Nitrosoanatabine (NAT)

The carcinogenicity of NAT in experimental animals has been evaluated previously (IARC, 1985).

Subcutaneous injection

Rat

Groups of male and female Fischer 344 rats, 9 weeks of age, received thrice-weekly subcutaneous injections of NAT in trioctanoin for 20 weeks (total doses, 1.0, 3.0 and 9.0 mmol/kg bw). Animals were killed when moribund or when only 20% of the animals were alive. A control group was injected with trioctanoin only. Major organs were fixed and examined microscopically. After treatment with NAT, 70–84% of the animals survived to 100 weeks. Lung adenocarcinomas developed in 1/21 male and 0/19 female rats injected with 1.0 mmol NAT; no primary lung tumours developed in rats injected with 3.0 or 9.0 mmol/kg NAT. At the 3.0 mmol/kg dose, 2/12 males had benign nasal cavity tumours. No nasal cavity tumours occurred at other doses. In trioctanoin controls, lung adenomas developed in 0/26 male and in 1/26 female rats (Hoffmann *et al.*, 1984).

4. Other Data Relevant to an Evaluation of Carcinogenicity and its Mechanisms

4.1 Absorption, distribution, metabolism and excretion

4.1.1 *Humans*

(*a*) *4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL)*

(i) *Absorption*

NNK has been detected in the saliva of snuff dippers, users of *khaini*, chewers of betel quid with tobacco and users of *toombak* (see Section 1.4.1(*a*)).

Absorption of NNK in smokeless tobacco users was demonstrated by the detection of its metabolites, NNAL and NNAL-Gluc, in plasma and urine and of NNAL-*N*-oxide in urine (Kresty *et al.*, 1996; Carmella *et al.*, 1997; Hecht, 2002; Hecht *et al.*, 2002). Similar results have been obtained in smokers, although only NNAL has been reported in plasma (Hecht *et al.*, 1999a; Hecht, 2002). NNAL-*N*-Gluc comprised 50 ± 25% of total NNAL-Gluc in the urine of smokers and 24 ± 12% in snuff-dippers (Carmella *et al.*, 2002). *Toombak* users excreted exceptionally high levels of NNAL and NNAL-Gluc (0.12–0.14 mg per day), which demonstrated a higher uptake of NNK by humans than of any other non-occupational carcinogen (Murphy *et al.*, 1994). Further information on NNAL and NNAL-Gluc in urine is presented in Section 4.1.1(*c*).

Absorption of NNK by nonsmokers exposed to secondhand cigarette smoke has been demonstrated by detection of NNAL and NNAL-Gluc in urine in several studies (Hecht, 2002).

NNAL, but not NNAL-Gluc, was detected in the amniotic fluid of mothers who smoked (Milunsky *et al.*, 2000). Both NNAL and NNAL-Gluc were detected in the urine of neonates born to mothers who smoked, but not in the urine of newborns of nonsmoking mothers (Lackmann *et al.*, 1999). These results indicate that NNK is converted to NNAL in the mother, and that NNAL crosses the placental barrier and is absorbed and metabolized to NNAL-Gluc in the late stages of fetal development.

(ii) *Distribution*

As noted above, NNAL and NNAL-Gluc have been detected in the plasma of smokeless tobacco users, and NNAL has been detected in plasma of smokers (Hecht *et al.*, 1999a, 2002). Pyridyloxobutyl–haemoglobin adducts derived from NNK and/or NNN have been detected in the blood of smokers, snuff-dippers, *toombak* users and nasal snuff users (Hecht, 1998). NNK was detected in the cervical mucus of smokers and its levels were significantly higher than those in nonsmokers (Prokopczyk *et al.*, 1997). NNK and NNAL were detected in pancreatic juice of smokers. Levels of NNK were significantly higher than in pancreatic juice from nonsmokers, and NNAL was detected more frequently in smokers than in nonsmokers (Prokopczyk *et al.*, 2002). DNA adducts of NNK and/or NNN have been detected in the lung tissue of smokers (Hecht, 1998). Haemoglobin and DNA adducts are discussed in more detail in Section 4.1.1(*c*).

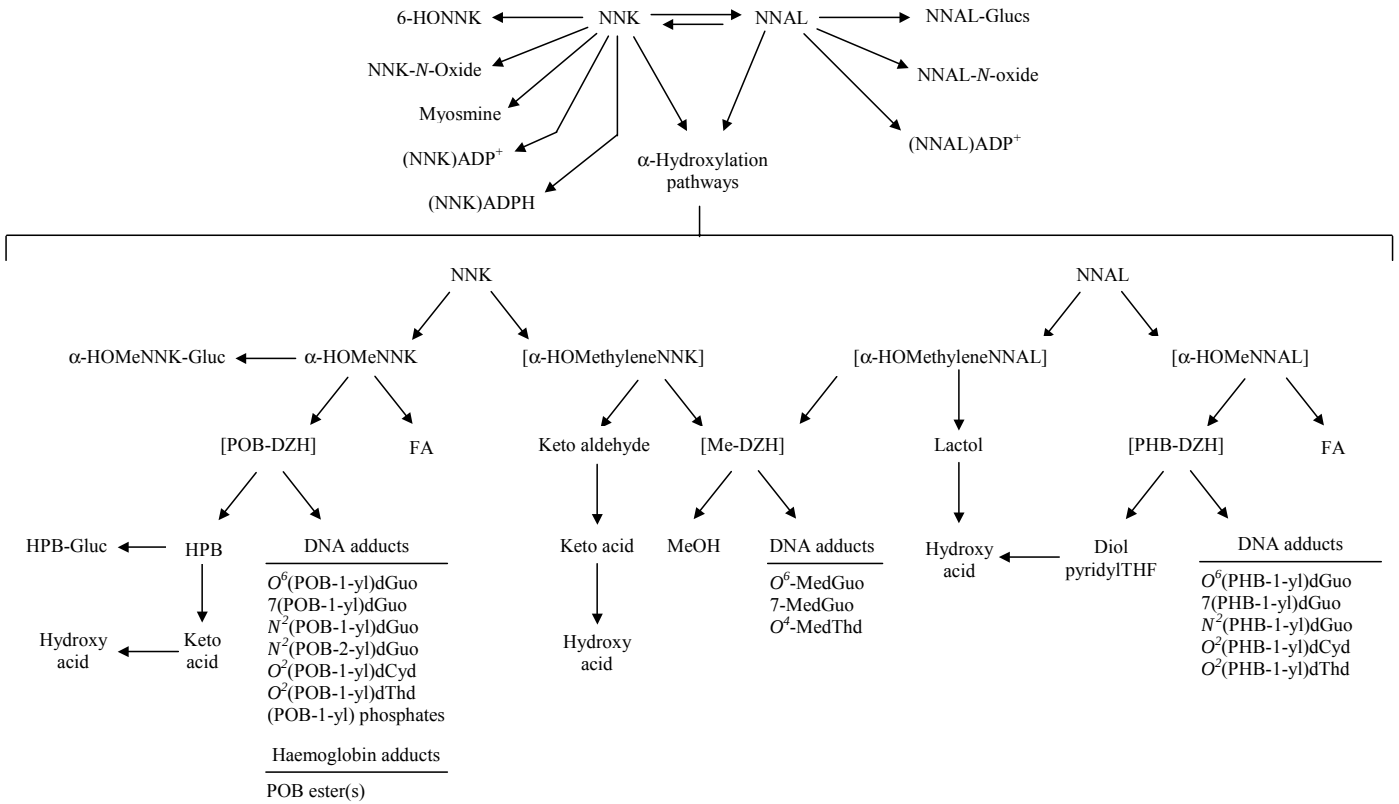
NNAL and NNAL-Gluc are excreted in the urine more slowly than would be expected, based on their structures, after cessation of smoking or smokeless tobacco use (Hecht *et al.*, 1999a, 2002). One week after smoking cessation, 34.5% of baseline NNAL plus NNAL-Gluc was detected in urine, whereas the corresponding values for the structurally related compounds cotinine and nicotine were 1.1 and 0.5%, respectively. Even 6 weeks after cessation, 7.6% of the original levels of NNAL plus NNAL-Gluc remained. The distribution half-life of NNAL and NNAL-Gluc was 3–4 days, while the elimination half-life was 40–45 days. Total body clearance of NNAL was estimated to be 61.4 ± 35.4 mL/min, and the volume of distribution in the β-phase was estimated to be 3800 ± 2100 L, which indicates substantial distribution into tissues (Hecht *et al.*, 1999a). After cessation of smokeless tobacco use, the distribution half-lives of NNAL (1.32 ± 0.85 versus 3.35 ± 1.86 days) and NNAL-Gluc (1.53 ± 1.22 versus 3.89 ± 2.43 days) were significantly shorter than those in smokers. There were no significant differences in the terminal half-lives. Ratios of (*S*)-NNAL:(*R*)-NNAL and (*S*)-NNAL-Gluc:(*R*)-NNAL-Gluc in urine were significantly (3.1–5.7 times) higher 7 days after cessation than at baseline in both smokeless tobacco users and smokers, which indicates stereoselective retention of (*S*)-NNAL in humans. From these results, the authors suggest that there is a receptor in the body, possibly in the lung, for (*S*)-NNAL (Hecht *et al.*, 2002).

(iii) *Metabolism*

Introduction

The metabolic pathways of NNK and NNAL and the modes of formation of their adducts are summarized in Figure 2. Structures of the NNK and NNAL metabolites are

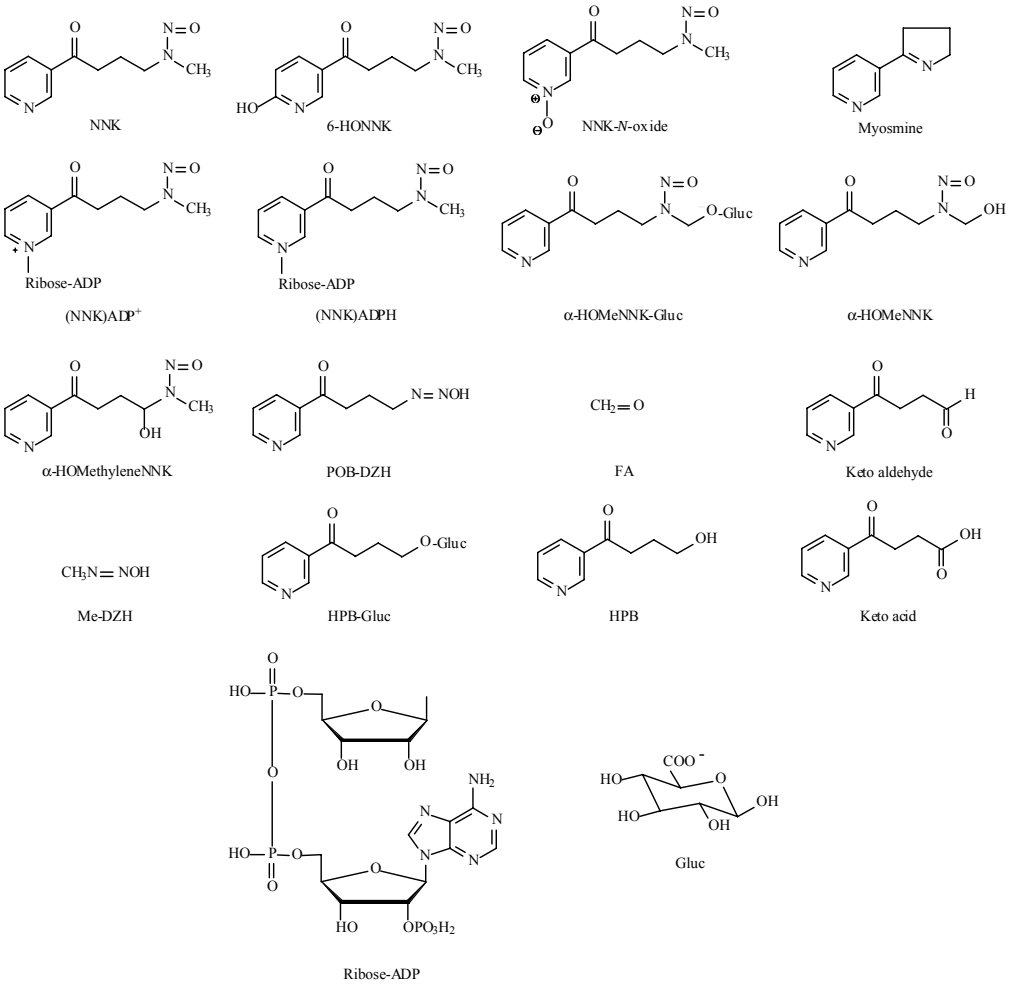
Figure 2. Metabolism of NNK and NNAL and formation of adducts, based on studies in laboratory animals and humans



ADP+, adenosine dinucleotide phosphate; ADPH, adenosine dinucleotide phosphate (reduced form); dCyd, deoxycytidine; dGuo, deoxyguanosine; dThd, deoxythymidine; DZH, diazohydroxide; Gluc, glucuronide; FA, formaldehyde; HO, hydroxy; HOMe, hydroxymethyl; HOMethylene, hydroxymethylene; HPB, 4-hydroxy-1-(3-pyridyl)-1-butanone or keto alcohol; MedGuo, methyldeoxyguanosine; Me-DZH, methaneDZH; MedThd, methyldeoxythymidine; MeOH, methanol; NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; PHB, 4-hydroxy-4-(3-pyridyl)-1-butyl; PHB-DZH, 4-hydroxy-4-(3-pyridyl)-1-butaneDZH; POB, 4-oxo-4-(3-pyridyl)-1-butyl or pyridyloxobutyl; POB-DZH, 4-oxo-4-(3-pyridyl)-1-butaneDZH; THF, tetrahydrofuran

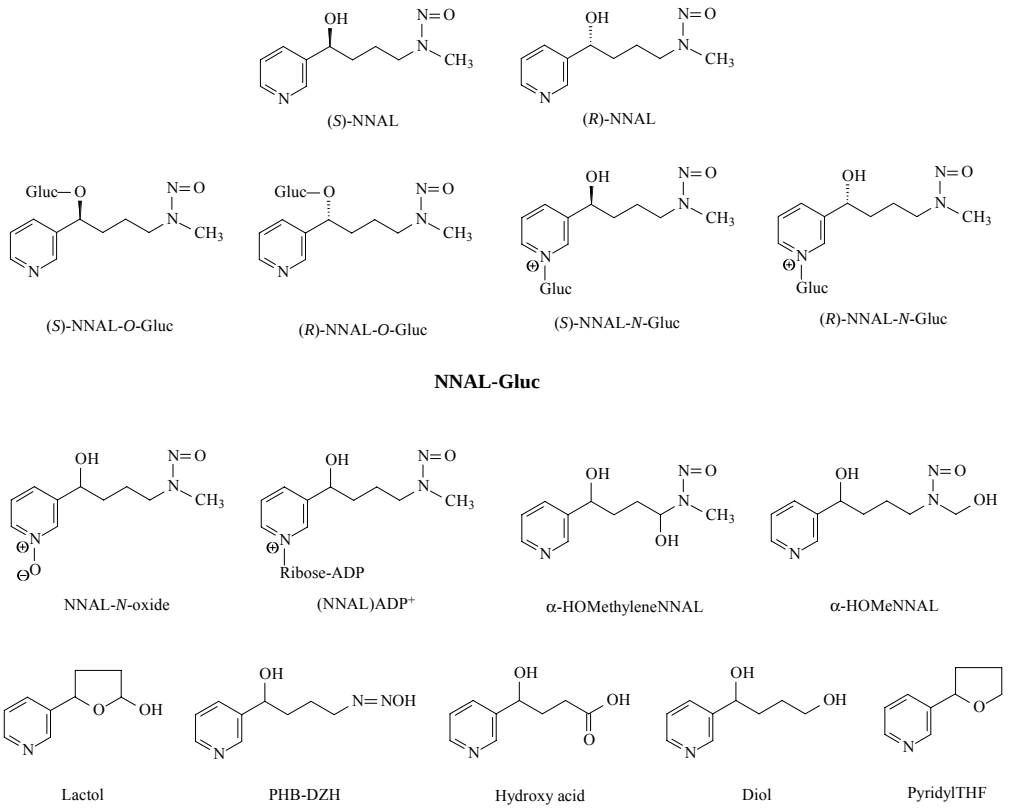
illustrated in Figures 3 and 4, and structures of the adducts are shown in Figure 5. This information is based on studies *in vitro*, in laboratory animals and in humans. Specific pathways that have been observed in in-vitro studies with human tissues or enzymes or in humans are discussed below (see Hecht, 1998).

Figure 3. Structures of NNK and metabolites



DZH, diazohydroxide; FA, formaldehyde; Gluc, glucuronide; HO, hydroxy; HOMe, hydroxymethyl; HOMethylene, hydroxymethylene; HPB, 4-hydroxy-1-(3-pyridyl)-1-butanone or keto alcohol; Me-DZH, methaneDZH; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; (NNK)ADP+, (NNK)adenosine dinucleotide phosphate; (NNK)ADPH, (NNK)adenosine dinucleotide phosphate (reduced form); POB-DZH, 4-oxo-4-(3-pyridyl)-1-butaneDZH

Figure 4. Structures of NNAL and metabolites

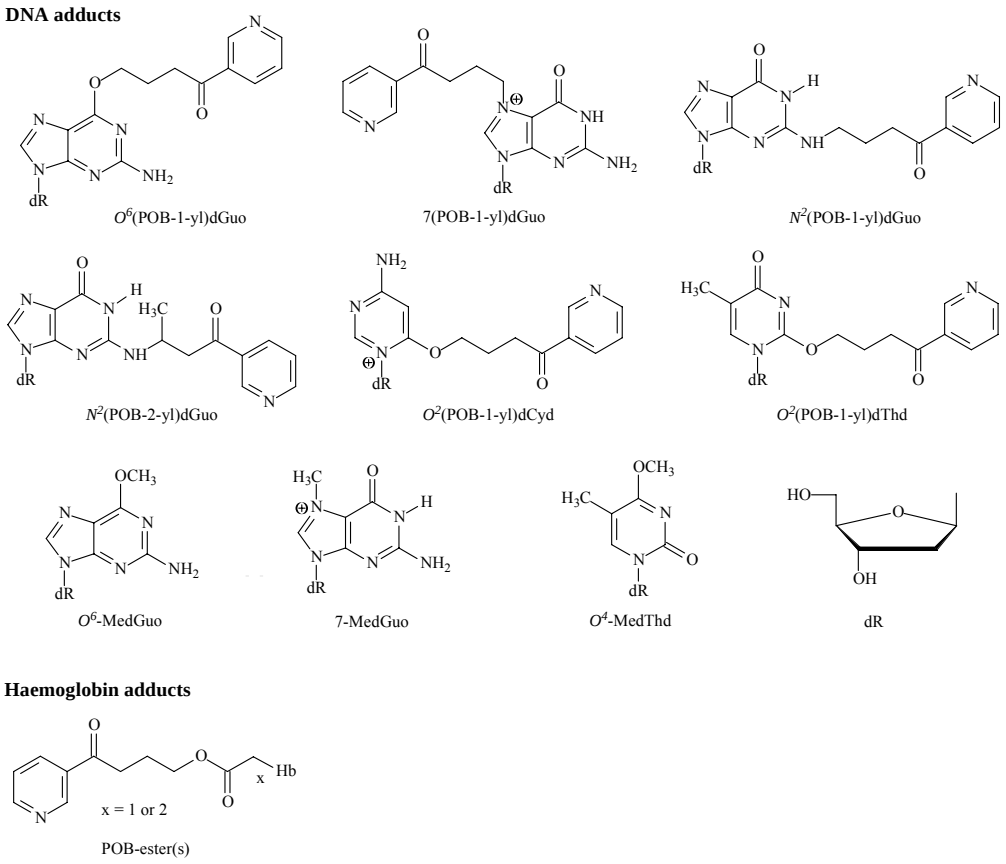


DZH, diazohydroxide; Gluc, glucuronide; HOMe, hydroxymethyl; HOMethylene, hydroxymethylene; NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; (NNAL)ADP+, (NNAL)adenosine dinucleotide phosphate; PHB-DZH, 4-hydroxy-4-(3-pyridyl)-1-butaneDZH; THF, tetrahydrofuran
See Figure 3 for structures of Gluc and ribose-ADP.

NNK can be converted to the pyridine oxidation products 4-(methylnitrosamino)-1-[3-(6-hydroxypyridyl)]-1-butanone (6-HONNK) and NNK-*N*-oxide. Denitrosation of NNK followed by oxidation produces myosmine. NNK can replace nicotinamide in $NADP^+$ or NADPH, to yield NNK adenosine dinucleotide phosphate ((NNK)ADP^+) and (NNK)ADPH (reduced form). Carbonyl reduction of NNK produces NNAL which can be conjugated by glucuronidation giving four diastereomers of NNAL-Gluc (Figure 4): two isomers of 4-(methylnitrosamino)-1-(3-pyridyl)-1-(*O*-β-D-glucopyranuronosyl)butane (NNAL-*O*-Gluc) and two isomers of 4-(methylnitrosamino)-1-(3-pyridyl-*N*-β-D-glucopyranuronosyl)-1-butanolonium inner salt (NNAL-*N*-Gluc). NNAL is also converted to NNAL-*N*-oxide and NNAL(ADP^+).

α-Hydroxylation of NNK and NNAL leads to DNA and haemoglobin adducts. Hydroxylation of the NNK methyl group gives 4-(hydroxymethylnitrosamino)-1-(3-pyridyl)-1-

Figure 5. Structures of DNA adducts and haemoglobin adducts derived from NNK



dCyd, deoxycytidine; dGuo, deoxyguanosine; dThd, deoxythymidine; dR, deoxyribose; Me, methyl; POB, 4-oxo-4-(3-pyridyl)-1-butyl or pyridyloxobutyl

Adducts derived from NNAL have the same structures except that an hydroxy group replaces the carbonyl group in the adducted portion of the molecule.

butanone (α-HOMeNNK) which can be conjugated as a glucuronide, α-HOMeNNK-Gluc. α-HOMeNNK spontaneously decomposes to 4-oxo-4-(3-pyridyl)-1-butanediazohydroxide (POB-DZH) and formaldehyde. POB-DZH reacts with water to give 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB) which can be conjugated as its glucuronide, HPB-Gluc. POB-DZH also reacts with DNA and haemoglobin to produce a series of adducts (Figures 2 and 5). α-Hydroxylation of the NNK methylene group produces 4-(methylnitrosamino)-1-(3-pyridyl)-1-(4-hydroxy)butanone (α-HOMethyleneNNK). This metabolite spontaneously decomposes to 4-(3-pyridyl)-4-oxobutanal (keto aldehyde) and methanediazohydroxide (Me-DZH). Keto aldehyde is further metabolized to 4-(3-pyridyl)-4-oxobutanoic acid (keto acid) which in turn can be converted to 4-hydroxy-4-(3-pyridyl)butanoic acid (hydroxy acid).

Me-DZH reacts with water to yield methanol and with DNA to produce methyl adducts as shown in Figures 2 and 5. NNAL similarly undergoes α-hydroxylation at its methylene group to yield 4-(methylnitrosamino)-1-(3-pyridyl)-1-(4-hydroxy)butanol (α-HOMethyleneNNAL) and at its methyl group to yield 4-(hydroxymethylnitrosamino)-1-(3-pyridyl)-1-butanol (α-HOMeNNAL). α-HOMethyleneNNAL spontaneously decomposes to Me-DZH and 5-(3-pyridyl)-2-hydroxytetrahydrofuran (lactol), which can be converted to hydroxy acid. α-HOMeNNAL spontaneously decomposes to 4-hydroxy-4-(3-pyridyl)-1-butanediazohydroxide (PHB-DZH) and formaldehyde. PHB-DZH reacts with water to yield 4-(3-pyridyl)butane-1,4-diol (diol), cyclizes to 2-(3-pyridyl)tetrahydrofuran (pyridylTHF) and reacts with DNA and haemoglobin to produce the adducts shown in Figures 2 and 5.

In-vitro studies in human tissues and cells

NNK

A variety of human tissues that includes cultured bronchus, peripheral lung, trachea, buccal mucosa, oesophagus and bladder metabolize NNK (Castonguay *et al.*, 1983c; Hecht, 1998), as do microsomes from human tissues such as liver, lung, cervix, placenta and pancreas (Hecht, 1998; Jalas *et al.*, 2005). A common observation in all of these studies is extensive conversion to NNAL. NNK is converted to NNAL in cultured human tissues (50–80%) (Castonguay *et al.*, 1983c), human red blood cells (Murphy & Coletta, 1993) and human liver microsomes and cytosol (18 and 25%, respectively). The enantiomeric composition in the liver was 64% (*S*)-NNAL in microsomes and 90% (*S*)-NNAL in cytosol (Upadhyaya *et al.*, 2000). Human cervical cells and human cervical microsomes formed (*R*)-NNAL while human cervical cytosol produced mainly (*S*)-NNAL (Prokopczyk *et al.*, 2001). Five enzymes that mediate conversion of NNK to NNAL in humans have been identified to date: microsomal 11β-hydroxysteroid dehydrogenase type 1 and cytosolic carbonyl reductase, which both belong to the superfamily of the short-chain dehydrogenases/reductases, and three members of the aldo-keto reductase superfamily (Maser, 2004).

Microsomes from human liver catalyse the α-hydroxylation of NNK to keto alcohol and keto aldehyde and the pyridine-*N*-oxidation to NNK-*N*-oxide. The rates of these reactions are lower than that of carbonyl reduction to NNAL (Smith *et al.*, 1992a; Patten *et al.*, 1996; Staretz *et al.*, 1997a; Jalas *et al.*, 2005) and reported Michaelis constant (K_m) values are relatively high. Correlation studies and the use of chemical inhibitors and inhibitory antibodies have suggested a role for cytochrome P450s (CYPs) 1A2, 2A6 and 3A4 in the formation of keto alcohol or keto aldehyde (Smith *et al.*, 1992a; Patten *et al.*, 1996; Hecht, 1998; Jalas *et al.*, 2005). Microsomes from human lung catalyse the conversion of NNK to keto aldehyde, hydroxy acid and NNK-*N*-oxide. The rate of metabolism of NNK to keto aldehyde was very low (less than 0.05 pmol/mg/min) whereas the rate in liver microsomes was 1–5 pmol/mg/min (Smith *et al.*, 1992a). One study suggested that lipoxygenase may be involved in the metabolism of NNK by human lung, but another study demonstrated the contrary (Smith *et al.*, 1995; Bedard *et al.*, 2002). The contribution of CYP2A6 and/or CYP2A13, as well as CYP2B6 to the α-hydroxylation of NNK and NNAL by human lung samples were suggested by selected chemical and antibody inhibi-

tion in some subjects (Jalas *et al.*, 2003a; Smith *et al.*, 2003). One of the difficulties in studying xenobiotic metabolism in human lung microsomes is the low level of CYP activity. CYP levels in lung microsomes are reported to be between 1 and 10 pmol/mg microsomal protein (Shimada *et al.*, 1992; Guengerich, 1993); the average concentration in 60 liver microsomal samples was 344 ± 167 pmol/mg hepatic microsomal protein (Shimada *et al.*, 1994). CYP-catalysed activity levels and mRNA levels are also much lower in the lung than in the liver (Shimada *et al.*, 1996; Ding & Kaminsky, 2003). However, CYPs have been reported to be expressed in particular regions of the lung which may result in higher localized concentrations (Anttila *et al.*, 1997; Hukkanen *et al.*, 2002). A diffusible inhibitor of CYP activity has been reported to be present in human lung microsome preparations; it was also reported that 7-ethoxycoumarin-*O*-deethylase activity in rat lung microsomes was inhibited by pre-incubation with human lung microsomes (Lorenz *et al.*, 1979).

Human fetal nasal microsomes metabolized NNK by α-hydroxylation to HPB and keto aldehyde with low K_m (6.7 ± 0.8 μM and 6.5 ± 1.1 μM, respectively) and antibody inhibition studies indicated that CYP2A13 (K_m, 2.8–11.3 μM; Table 11) was involved (Wong *et al.*, 2005a).

CYPs are the major catalysts of NNK α-hydroxylation in humans and rodents. Table 11 summarizes the steady-state kinetic parameters for CYP-mediated NNK metabolism (Jalas *et al.*, 2005). Based on the K_m data in Table 11, the relative efficiencies in NNK metabolism by human CYP are (from greatest catalyst to least): 2A13 > 2B6 > 2A6 > 1A2 ~ 1A1 > 2D6 ~ 2E1 ~ 3A4. Similar results are obtained when the ratio maximum velocity (V_{max})/K_m is considered. The actual involvement of these enzymes in NNK metabolism *in vivo* depends on many factors that include relative expression levels, the amount of CYP oxido-reductase expressed in a given tissue, tissue localization and inducibility of individual CYPs, and the concentration of NNK in human tissues. Among hepatic CYPs, 2B6 has the highest affinity for NNK. However, low levels of this enzyme are present in most liver samples (Gervot *et al.*, 1999). CYP2A6 is also present at relatively low levels, and accounts for < 1% to 4% of the total CYP content (Shimada *et al.*, 1996). Levels of CYP1A2 are four- to 20-fold higher than those of CYP2A6 (Shimada *et al.*, 1996). Therefore, despite its somewhat higher K_m and lower V_{max}/K_m, CYP1A2 is most probably as important a catalyst of NNK α-hydroxylation in human liver as CYP2A6. CYP3A4 may also play a role in hepatic NNK α-hydroxylation, since it is often present at concentrations that are 10–50 times greater than those of CYP2A6 (Shimada *et al.*, 1994). It is not possible to rule out completely the presence of CYP2A13, the best known human catalyst of NNK metabolism, in the liver. However, the very low hepatic mRNA levels of CYP2A13 relative to CYP2A6 suggest that, if this enzyme is present, it is so at very low levels. Results to date do not identify that any single CYP in the liver is a key player in NNK activation. Several enzymes, including CYP1A2, CYP2A6, CYP2B6 and CYP3A4, clearly play a role. The relative contribution of any one of these CYPs varies among individuals, and their relative abundance and catalytic efficiencies suggest that rarely, if ever, is one of them the dominant catalyst (Jalas *et al.*, 2005).

Table 11. Steady-state kinetic parameters for cytochrome P450 (CYP)-mediated NNK metabolism

Species/enzyme	NNK concentration range (μM)	Metabolite	Kinetic parameters			Expression system	Reference
			V_{max}[a]	K_m (μM)	V_{max}/K_m[b]		
Human CYP1A1	1–500	Keto aldehyde	4.44 ± 0.41	1400 ± 148	$3.2 \times 10^{-3} \pm 5 \times 10^{-4}$	Gentest Supersomes[c]	Smith *et al.* (1995)
		HPB	0.824 ± 0.118	371 ± 6	$2.2 \times 10^{-3} \pm 3 \times 10^{-4}$		
Human CYP1A2	1–1000	Keto aldehyde	0.51 ± 0.04	1180 ± 60	$4.3 \times 10^{-4} \pm 4 \times 10^{-5}$	Purified, reconstituted protein	Smith *et al.* (1996)
		HPB	1.7 ± 0.05	380 ± 30	$4.5 \times 10^{-3} \pm 4 \times 10^{-4}$		
		HPB	1.96 ± 0.07	400 ± 10	$4.9 \times 10^{-3} \pm 2 \times 10^{-4}$	as above, DMSO control	
			2.09 ± 0.20	760 ± 10	$2.8 \times 10^{-3} \pm 3 \times 10^{-4}$	as above, but with 50 nM PEITC added	
			2.06 ± 0.21	820 ± 20	$2.5 \times 10^{-3} \pm 3 \times 10^{-4}$	as above, but with 100 nM PEITC added	
			2.05 ± 0.25	1240 ± 70	$1.7 \times 10^{-3} \pm 2 \times 10^{-4}$	as above, but with 200 nM PEITC added	
Human CYP1A2[d]	10–350	HPB	4.2 ± 0.2	309 ± 16	0.014 ± 0.001	Hep G2 cell lysate	Smith *et al.* (1992a)
Human CYP2A6	5–2000	Keto aldehyde	0.437	392	1.11×10^{-3}	Baculovirus-infected *Spodoptera frugiperda* (Sf9) cells	Patten *et al.* (1996)
		HPB	0.163	349	4.67×10^{-4}		
Human CYP2A6 (+ b_5)[e]	5–2000	Keto aldehyde	1.03	118	8.73×10^{-3}	Baculovirus-infected Sf9 cells	Patten *et al.* (1996)
		HPB	0.419	141	2.97×10^{-3}		
Human CYP2A13	2–160	Keto aldehyde	4.1 ± 0.6	11.3 ± 3.5	0.36 ± 0.12	Baculovirus-infected Sf9 cells	Su *et al.* (2000)
		HPB	1.2 ± 0.2	13.1 ± 5.1	0.092 ± 0.039		
Human CYP2A13	2–100	Keto aldehyde	14.5 ± 0.8	4.6 ± 0.4	3.2 ± 0.3	Purified, reconstituted protein	Zhang *et al.* (2002)
		HPB	5.7 ± 0.7	2.8 ± 0.5	2.0 ± 0.4		
Human CYP2A13 (Arg 257 Cys)	2–100	Keto aldehyde	8.4 ± 0.8	6.2 ± 0.7	1.4 ± 0.2	Purified, reconstituted protein	Zhang *et al.* (2002)
		HPB	3.2 ± 0.5	4.8 ± 1.0	0.67 ± 0.17		
Human CYP2A13	0.25–50	Keto aldehyde	13.8 ± 0.8	3.6 ± 0.7	3.9 ± 0.8	Baculovirus-infected Sf9 cells	Jalas *et al.* (2003a)
		HPB	4.6 ± 0.2	3.2 ± 0.5	1.4 ± 0.2		

Table 11 (contd)

Species/enzyme	NNK concentration range (μM)	Metabolite	Kinetic parameters			Expression system	Reference
			V_{max}[a]	K_m (μM)	V_{max}/K_m[b]		
Human CYP2B6	2.5–150	Keto aldehyde and HPB[f]	0.18 ± 0.01	33 ± 0.7	$5.5 \times 10^{-3} \pm 3 \times 10^{-4}$	BD Biosciences Discovery Labware Supersomes	Dicke *et al.* (2005)
Human CYP2D6	5–2000	Keto aldehyde HPB	0.105 4.01	1061 5525	9.9×10^{-5} 7.3×10^{-4}	Baculovirus-infected Sf9 cells	Patten *et al.* (1996)
Human CYP2D6	5–2000	Keto aldehyde HPB	0.13 6.04	1075 5632	2×10^{-4} 1×10^{-3}	Chinese hamster ovary cells	Patten *et al.* (1996)
Human CYP2E1 (+ b_5)[e]	5–2000	Keto aldehyde HPB	0.026 1.17	720 3334	3.6×10^{-5} 3.5×10^{-4}	Baculovirus-infected Sf9 cells	Patten *et al.* (1996)
Human CYP3A4	5–8000	Keto aldehyde HPB	0.787 0.086	3091 1125	2.6×10^{-4} 7.6×10^{-5}	Chinese hamster ovary cells	Patten *et al.* (1996)
Rabbit CYP2A10/2A11	2.9–154	Keto aldehyde HPB	1.38 1.30	15 9.0	0.092 0.14	Purified, reconstituted protein	Hong *et al.* (1992)
Rabbit CYP2A10/2A11 (+ b_5)[e]	2.9–154	Keto aldehyde HPB	0.849 0.575	28.6 16.3	0.0297 0.0353	Purified, reconstituted protein	Hong *et al.* (1992)
Rabbit CYP2A10/2A11 (+ 80 μM nicotine)	2.9–154	Keto aldehyde HPB	1.33 1.26	40.2 29.5	0.033 0.043	Purified, reconstituted protein	Hong *et al.* (1992)
Rabbit CYP2G1	2.9–154	Keto aldehyde HPB	0.735 ND	186	3.95×10^{-3}	Purified, reconstituted protein	Hong *et al.* (1992)
Rat CYP1A1	1–5000	Keto aldehyde HPB	2.2 ± 0.1 0.68 ± 0.03	180 ± 34 140 ± 26	0.013 ± 0.002 $4.9 \times 10^{-3} \pm 9 \times 10^{-4}$	Gentest Supersomes	Jalas *et al.* (2005)
Rat CYP1A2	1–5000	Keto aldehyde HPB	5.0 ± 0.1 6.1 ± 0.09	180 ± 22 200 ± 11	0.028 ± 0.003 0.034 ± 0.002	Gentest Supersomes	Jalas *et al.* (2005)
Rat CYP2A3	0.25–50	Keto aldehyde HPB	10.8 ± 0.4 8.2 ± 0.3	4.6 ± 0.5 4.9 ± 0.5	2.3 ± 0.3 1.7 ± 0.2	Baculovirus-infected Sf9 cells	Jalas *et al.* (2003a)

Table 11 (contd)

Species/enzyme	NNK concentration range (μM)	Metabolite	Kinetic parameters			Expression system	Reference
			V_{max} [a]	K_m (μM)	V_{max}/K_m [b]		
Rat CYP2B1	10–1300	Keto aldehyde HPB NNK-*N*-oxide	0.0897 ± 0.003 0.333 ± 0.016 0.295 ± 0.006	191 ± 20 318 ± 5 131 ± 10	$4.70 \times 10^{-4} \pm 5.2 \times 10^{-5}$ $1.05 \times 10^{-3} \pm 5 \times 10^{-5}$ $2.25 \times 10^{-3} \pm 1.7 \times 10^{-4}$	Purified, reconstituted protein	Guo *et al.* (1991a)
Rat CYP2C6	1–5000	Keto aldehyde HPB NNK-*N*-oxide	2.5 ± 0.1 16 ± 0.5 1.5 ± 0.06	1300 ± 130 1400 ± 89 1100 ± 110	$1.9 \times 10^{-3} \pm 2 \times 10^{-4}$ 0.011 ± 0.0008 $1.4 \times 10^{-3} \pm 4 \times 10^{-4}$	Gentest Supersomes	Jalas *et al.* (2005)
Mouse CYP2A4	1–5000	Keto aldehyde	190 ± 23	3900 ± 995	0.049 ± 0.014	Baculovirus-infected Sf9 cells	Felicia *et al.* (2000)
Mouse CYP2A4	0.5–3000	Keto aldehyde HPB	7.3 ± 0.4 1.8 ± 0.1	97 ± 19 67 ± 17	0.075 ± 0.015 0.027 ± 0.007	Baculovirus-infected Sf9 cells	Jalas *et al.* (2003b)
Mouse CYP2A5	0.25–100	HPB	4.0 ± 0.4	1.5 ± 0.6	2.7 ± 1.8	Baculovirus-infected Sf9 cells	Felicia *et al.* (2000)
Mouse CYP2A5	0.25–50	Keto aldehyde HPB	2.0 ± 0.1 6.5 ± 0.2	4.3 ± 0.8 4.5 ± 0.4	0.47 ± 0.09 1.4 ± 0.1	Baculovirus-infected Sf9 cells	Jalas *et al.* (2003b)

Adapted from Jalas *et al.* (2005)

DMSO, dimethyl sulfoxide; HPB, 4-hydroxy-1-(3-pyridyl)-1-butanone; K_m, Michaelis constant; NNK, 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone; PEITC, phenethyl isothiocyanate; ND, not detected; OPB, 4-oxo-4-(3-pyridyl)butanol or keto aldehyde; V_{max}, maximum velocity

[a] Units are pmol product/min/(pmol CYP).

[b] Units are pmol product/min/(pmol CYP)/μM.

[c] V_{max} units were converted using the published CYP concentration of 34 pmol CYP/(mg protein) (Smith *et al.*, 1995). Supersomes that contain co-expressed CYP and oxidoreductase were purchased from Gentest (Woburn, MA).

[d] The V_{max} value was computed based on a CYP1A2 concentration of 13 pmol/(mg protein); this is an upper limit, the lowest possible V_{max} value is 3.1 ± 0.1 pmol/min/(pmol CYP) based on 18 pmol/(mg protein). See Aoyama *et al.* (1990).

[e] Cytochrome b_5 was added at a 3:1 b_5:CYP molar ratio. b5:CYP molar ratio is corrected according to the original reference (Patten *et al.*, 1996).

[f] HBP:keto aldehyde (OPB) ratio ≈ 10:1

In the lung, CYP2A13 may be the key catalyst of NNK α-hydroxylation (Su *et al.*, 2000; Jalas *et al.*, 2003a). However, the level of CYP2A13 protein in the lung is unknown. The relative levels of human lung CYPs are not well characterized. CYP1A1 (when induced in smokers) and CYP1B1 are probably present at relatively high levels in this tissue (Shimada *et al.*, 1992; Spivack *et al.*, 2001). CYP1A1 is a catalyst of NNK metabolism but is much less efficient than CYP2A13 (Table 11). Metabolism of NNK by CYP1B1 has not been studied. Based on reported expression levels, other enzymes that may contribute to NNK activation in the lung are CYP2B6 and CYP3A5 (Table 11; Hukkanen *et al.*, 2002; Smith *et al.*, 2003).The kinetic parameters for CYP3A5-catalysed NNK metabolism have not been determined.

NNAL

Human liver microsomes convert NNAL to NNAL-*O*-Gluc and NNAL-*N*-Gluc (Wiener *et al.*, 2004a). The hepatic enzymes UGT2B7 and UGT1A9 appear to be important catalysts for conversion of NNAL to NNAL-*O*-Gluc while UGT1A4 plays a significant role in the formation of NNAL-*N*-Gluc (Ren *et al.*, 2000; Wiener *et al.*, 2004a). Large variations in the ability to glucuronidate NNAL have been observed among liver microsomal specimens from different humans. Polymorphisms in the *UGT1A4* and *UGT2B7* genes were associated with altered levels of NNAL glucuronidation in these specimens (Wiener *et al.*, 2004b). Thus, interindividual differences in the conversion of NNK to NNAL and of NNAL to NNAL-Gluc could be important in determining individual susceptibility to the carcinogenic effects of NNK.

Human liver microsomes convert NNAL to lactol, diol, hydroxy acid, NNAL-*N*-oxide and NNK. Conversion to NNK occurs at the fastest rate (Staretz *et al.*, 1997b). There was no significant difference in the rates of metabolism of (*S*)- and (*R*)-NNAL by human liver microsomes (Upadhyaya *et al.*, 2000). Human CYP2A13 catalyzed the conversion of racemic NNAL to lactol, diol and NNAL-*N*-oxide with K_m values of 36 ± 3, 40 ± 3 and 30 ± 7 μM, respectively (Jalas *et al.*, 2003a).

In-vivo studies

Overview

The metabolism of NNK in humans was definitively established by studies that demonstrated the presence of its metabolites in the urine of smokeless tobacco users, smokers and nonsmokers exposed to secondhand smoke. NNK metabolites identified and quantified to date are NNAL, NNAL-*O*-Gluc, NNAL-*N*-Gluc and NNAL-*N*-oxide. NNAL and NNAL-Gluc have also been detected in plasma. These metabolites have been reported only in individuals exposed to tobacco or tobacco smoke, and their levels are too high for them to derive from the small amounts of NNAL in tobacco products. The only possible source is NNK. In addition, haemoglobin and DNA adducts that could arise from either NNK or NNN have been quantified in human blood and tissues.

NNAL and NNAL-Gluc

Studies on NNAL and NNAL-Gluc in human urine have been reviewed (Hecht, 2002; IARC, 2004) (see also Section 1.4.1(*c*)). Mean levels reported in the urine of smokeless tobacco users, smokers and nonsmokers exposed to secondhand tobacco smoke are summarized in Table 12.

Table 12. Total NNAL in the urine of smokeless tobacco users, smokers and nonsmokers exposed to secondhand tobacco smoke

No. of subjects	pmol/mL	pmol/mg creatinine	nmol/24 h	Reference
Smokeless tobacco users				
7 *toombak* users	1270	NR	NR	Murphy *et al.* (1994)
39 smokeless tobacco users	NR	4.4	NR	Kresty *et al.* (1996)
13 smokeless tobacco users	4.22	3.55	6.60	Hecht *et al.* (2002)
10 snuff dippers	4.20	NR	NR	Carmella *et al.* (2002)
55 snuff dippers	NR	3.25	NR	Carmella *et al.* (2003)
41 snuff dippers	NR	3.0	NR	Hatsukami *et al.* (2004)
54 snuff dippers	NR	3.3	NR	Lemmonds *et al.* (2005)
Smokers				
11 smokers	NR	NR	11.4	Carmella *et al.* (1993)
61 smokers	NR	3.76	NR	Carmella *et al.* (1995)
11 smokers	NR	NR	3.28	Hecht *et al.* (1995)
20 smokers	NR	NR	3.22	Meger *et al.* (2000)
13 smokers	NR	3.90	NR	Taioli *et al.* (1997)
27 smokers	1.95	2.70	3.14	Hecht *et al.* (1999a)
18 smokers	NR	3.69	NR	Hurt *et al.* (2000)
10 smokers	1.22	NR	NR	Carmella *et al.* (2002)
99 smokers	NR	2.07	NR	Hecht *et al.* (2004a)
41 smokers	NR	2.60	NR	Carmella *et al.* (2003)
34 smokers	NR	3.5	NR	Hughes *et al.* (2004)
38 smokers	NR	2.3	NR	Hatsukami *et al.* (2004)
84 smokers	NR	1.6	NR	Hecht *et al.* (2004b)
7 smokers	2.36	NR	5.32	Byrd & Ogden (2003)
12 smokers	2.8	NR	NR	Breland *et al.* (2003)
11 smokers	NR	1.34	NR	Sellers *et al.* (2003)
Nonsmokers exposed to secondhand tobacco smoke				
5 exposed men	NR	NR	0.127	Hecht *et al.* (1993a)
9 exposed hospital workers	0.059	NR	NR	Parsons *et al.* (1998)
29 exposed nonsmokers	NR	NR	0.027	Meger *et al.* (2000)
23 exposed women	0.050	NR	NR	Anderson *et al.* (2001)
74 exposed children	0.056	NR	NR	Hecht *et al.* (2001)
16 casino patrons	0.033	0.037	NR	Anderson *et al.* (2003)
19 restaurant workers	0.066	0.070	0.107	Tulunay *et al.* (2005)
27 newborns of smoking mothers	0.14	NR	NR	Lackmann *et al.* (1999)

NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL); NR, not reported

Smokeless tobacco users

Snuff dippers/tobacco chewers in the USA excreted 6.6 nmol/24 h total NNAL (NNAL plus NNAL-Gluc) in urine (Hecht *et al.*, 2002) (Table 12). Swedish snuff users excreted 48% less total NNAL than snuff dippers who used products marketed in the USA (Hatsukami *et al.*, 2004). Assuming that total NNAL represents 20% of the NNK dose, uptake of NNK amounts to 33 nmol per day in snuff dippers/chewers and 17 nmol per day in Swedish snuff users. The total dose of NNK would be 4.8 μmol/kg bw over 30 years of snuff dipping/chewing and 2.5 μmol/kg bw over 30 years of *snus* use. Rats treated with 0.5 ppm of NNK in the drinking-water for 2 years (total dose, 73 μmol/kg bw) had an increased incidence of exocrine pancreatic tumours (Rivenson *et al.*, 1988) (see Section 3.1.4(*b*)). The total dose in the rat study was about 15–29 times higher than the estimated total human dose achieved over 30 years of snuff dipping/chewing or *snus* use. Lower doses of NNK have not been tested for induction of pancreatic tumours, but tumorigenicity of NNK in the rat pancreas is enhanced by a high-fat diet (Hoffmann *et al.*, 1993a).

Toombak users excreted an average of 1270 pmol/mL urine total NNAL, which was approximately 300 times higher than that excreted by snuff dippers/chewers or Swedish snuff users (4.2 pmol/mL) (Murphy *et al.*, 1994; Carmella *et al.*, 2002). Applying the above calculation, the 30-year total dose of NNK in *toombak* users would be about 1440 μmol/kg bw (298 mg/kg bw). NNN levels in the saliva of *toombak* users were approximately 30 times greater than those of NNK (Idris *et al.*, 1992). Thus, the corresponding NNN dose could be estimated as 43 200 μmol/kg bw (9.04 g/kg bw). These estimated total doses of NNK (1440 μmol/kg bw) and NNN (43 200 μmol/kg bw) can be compared with the total doses of NNK (240 μmol/kg bw) and NNN (1400 μmol/kg bw) which induced a significantly increased incidence of oral cavity tumours when swabbed repeatedly in the oral cavity of Fischer 344 rats (Hecht *et al.*, 1986b). Thus, 30-year *toombak* users are exposed to total doses of NNK and NNN that are approximately six and 31 times higher than those required to induce oral tumours in rats.

The ratio of (*S*)-NNAL-*O*-Gluc:(*R*)-NNAL-*O*-Gluc was 1.9 and that of NNAL-*O*-Gluc:NNAL-*N*-Gluc was 7.1 in the urine of *toombak* users (Murphy *et al.*, 1994; Carmella *et al.*, 2002) and 3.6 in the urine of snuff dippers (Carmella *et al.*, 2002). Free NNAL comprised 31% of total NNAL in the urine of *toombak* users (Murphy *et al.*, 1994) and 35% of total NNAL in the urine of snuff dippers (Carmella *et al.*, 2002).

When snuff dippers who used products marketed in the USA switched to a brand of Swedish snuff over a 4-week period, a statistically significant average reduction of about 48% in total urinary NNAL was observed. However, when snuff dippers switched to a nicotine patch for 4 weeks, there was a significantly greater reduction of about 90% in total NNAL, which showed that a switch to *snus* is inferior to abstention from smokeless tobacco with respect to NNK uptake (Hatsukami *et al.*, 2004).

In one study of snuff dippers and tobacco chewers, a significant association between urinary levels of total NNAL and the presence of oral leukoplakia was observed (Kresty *et al.*, 1996).

Smokers

Smokers excrete about 3.2 nmol/24 h total NNAL (Table 12). Assuming that total NNAL represents 20% of NNK dose, daily uptake of NNK would be 16.5 nmol, or 180 μmol (2.4 μmol/kg bw; 0.5 mg/kg bw) over 30 years of smoking. The lowest total dose of NNK shown to induce a significantly increased incidence of lung tumours in rats, as part of a dose–response relationship, was 1.8 mg/kg bw (8.7 μmol/kg bw), 3.6 times higher than the dose of a smoker (Belinsky *et al.*, 1990) (see Section 3.1.2(*a*)).

The enantiomeric distribution of NNAL in urine was 54% (*R*)-NNAL and 46% (*S*)-NNAL whereas the diastereomeric distribution of NNAL-Gluc was 68% (*R*)-NNAL-Gluc and 32% (*S*)-NNAL-Gluc (Carmella *et al.*, 1999). (*R*)-NNAL is the more tumorigenic NNAL enantiomer in A/J mice (Upadhyaya *et al.*, 1999). The ratio of NNAL-*O*-Gluc:NNAL-*N*-Gluc was 1.3 in smokers (Carmella *et al.*, 2002). Free NNAL comprised a mean of 38% of total NNAL in smokers, and gave an NNAL-Gluc:NNAL ratio of 1.6 (Carmella *et al.*, 2002). A wide range of NNAL-Gluc:NNAL ratios has been observed and there is evidence for a high ratio (6–11) phenotype in a minority of smokers (Carmella *et al.*, 1995).

Consistently, total NNAL correlates with total cotinine in smokers (Hecht, 2002), number of cigarettes per day and 1-hydroxypyrene in urine. The increase in total NNAL with number of cigarettes per day was not linear (Joseph *et al.*, 2005). As cotinine is a marker for the uptake of nicotine, total NNAL is a marker for the uptake of NNK.

Five recent studies have employed NNAL and NNAL-Gluc, as well as other biomarkers, to investigate approaches to tobacco harm reduction. Two concerned the effects of reducing numbers of cigarettes smoked per day and whether this would have a significant effect on the uptake of NNK. One study of 23 subjects found a moderate reduction of NNAL-Gluc and total NNAL (Hurt *et al.*, 2000). A second study of 102 smokers examined NNAL and NNAL-Gluc in the urine of smokers who reduced their smoking by up to 75% over a 6-week period. Statistically significant reductions in NNAL, NNAL-Gluc and total NNAL were observed at most intervals, but the observed decreases were generally modest, were always proportionally lower than the reductions in the number of cigarettes smoked per day and were sometimes transient (Hecht *et al.*, 2004a). Apparently, smokers compensate in their smoking behaviour when they smoke fewer cigarettes per day, and thereby decrease the expected reduction in carcinogen uptake. Three studies examined the effects of switching to brands with lower NNK delivery, as measured by standardized machine methods. In one study of the Omni cigarette, which, according to machine measurement, delivers less NNK than traditional brands, no significant decrease in the level of total NNAL was observed (Hughes *et al.*, 2004). A second study of Omni did demonstrate a significant reduction in total NNAL compared with the smokers' usual brand, but less than that achieved by cessation with medicinal nicotine (Hatsukami *et al.*, 2004). A significant reduction in total NNAL was also observed in smokers who switched to the Advance cigarette (Breland *et al.*, 2003).

Levels of NNAL and NNAL-Gluc were measured in the urine of 84 Singapore Chinese smokers, who were interviewed about their intake of cruciferous vegetables (Hecht *et al.*,

2004b). There was a significant correlation between increased consumption of glucobrassicins (precursors of indole-3-carbinols) from these vegetables and decreased levels of NNAL in the urine, after adjustment for number of cigarettes smoked per day; similar trends were observed for NNAL-Gluc and total NNAL. These results are consistent with those of previous studies that demonstrated that indole-3-carbinol, an in-vivo hydrolysis product of glucobrassicins, decreased the levels of urinary NNAL, probably by inducing hepatic metabolism of NNK (Morse *et al.*, 1990a; Taioli *et al.*, 1997).

Urine samples from 175 smokers of regular, light or ultra-light cigarettes were analysed for total NNAL (Hecht *et al.*, 2005). There were no statistically significant differences in urinary levels of total NNAL among smokers of these types of cigarettes, and no correlation between levels of tar and total NNAL. These results are consistent with epidemiological studies that showed no difference in the risk for lung cancer among smokers of different types of cigarettes (Burns *et al.*, 2001; Harris *et al.*, 2004).

Nonsmokers exposed to secondhand tobacco smoke

Levels of total NNAL in the urine of nonsmokers exposed to secondhand tobacco smoke are typically about 1–5% of those in smokers (Table 12). Correlations between levels of urinary cotinine and total NNAL have consistently been observed in studies of nonsmokers exposed to secondhand tobacco smoke (Hecht, 2002; IARC, 2004). Most studies on NNAL in the urine of nonsmokers have been reviewed in a previous monograph which concluded: "The data demonstrating uptake of NNK, a lung carcinogen in rodents, by nonsmokers are supportive of a causal link between exposure to secondhand tobacco smoke and development of lung cancer" (IARC, 2004). Two studies have appeared since that time. In one, total NNAL was quantified before and after a 4-h visit to a gambling casino where smoking was allowed. Paired samples showed statistically significant mean increases in total cotinine (cotinine plus its glucuronide) and total NNAL in urine after the visit (Anderson *et al.*, 2003). A second study examined the uptake of total nicotine, total cotinine and total NNAL in nonsmokers who worked in restaurants and bars that permit smoking (Tulunay *et al.*, 2005). Urine samples were collected for 24 h on working and non-working days. The results showed significant increases in urinary levels of total nicotine, total cotinine and total NNAL on working days compared with non-working days.

NNAL-N-*oxide*

Levels of NNAL-*N*-oxide ranged from 0.06 to 1.4 pmol/mg creatinine (mean, 0.53 pmol/mg creatinine) in the urine of smokers and from 0.02 to 1.2 pmol/mg creatinine (mean, 0.41 pmol/mg creatinine) in the urine of smokeless tobacco users. NNK-*N*-oxide was not detected in the urine. The amounts of NNAL-*N*-oxide in urine were about 20 and 50% of the amounts of NNAL-Gluc and NNAL, respectively. Thus, pyridine-*N*-oxidation is less important than glucuronidation as a detoxification pathway for NNK and NNAL in humans (Carmella *et al.*, 1997).

Haemoglobin adducts

Haemoglobin adducts of NNK and NNN are formed upon reaction of a common intermediate, POB-DZH, with aspartate or glutamate in haemoglobin. In the case of NNK, POB-DZH is generated by CYP-mediated hydroxylation of the methyl group to give α-HOMeNNK (Figures 2 and 5). The POB-aspartate and -glutamate esters in haemoglobin can readily be hydrolysed by base treatment to release HPB, which can be quantified by gas chromatography–mass spectrometry (Carmella *et al.*, 1990a; Hecht, 1998). The presence of HPB-releasing haemoglobin adducts in humans provides strong evidence for the metabolic activation of NNK and/or NNN, although another possible source — nitrosation of myosmine — has been proposed (Wilp *et al.*, 2002).

The highest levels of HPB-releasing haemoglobin adducts have been found in smokeless tobacco users. Mean levels (fmol/g haemoglobin) were 517 in snuff-dippers, 236 in nasal snuff users and 148 in *toombak* users (Carmella *et al.*, 1990a; Falter *et al.*, 1994; Murphy *et al.*, 1994). Lower levels were reported in smokers. Mean levels (fmol/g haemoglobin) in smokers and nonsmokers in four studies were 79.6 and 29.3 (Carmella *et al.*, 1990a), 54.7 and 26.7 (Branner *et al.*, 1998), 61 and 34 (Falter *et al.*, 1994) and 26 and 19 (Atawodi *et al.*, 1998). Levels of HPB-releasing haemoglobin adducts were not higher in nonsmokers exposed to secondhand tobacco smoke than in non-exposed nonsmokers (Branner *et al.*, 1998).

DNA adducts

NNK can form adducts by two α-hydroxylation pathways (Figure 2). α-Methyl hydroxylation produces α-HOMeNNK, which spontaneously decomposes to POB-DZH. POB-DZH can also be formed by 2′-hydroxylation of NNN. POB-DZH reacts with DNA to give a variety of adducts (Figures 2 and 5), some of which release HPB upon acid or neutral thermal hydrolysis. POB-DZH-derived adducts in DNA can be quantified by treating the DNA with acid and measuring released HPB by gas chromatography–mass spectrometry. α-Methylene hydroxylation of NNK or NNAL ultimately produces Me-DZH, which can react with DNA to produce O^6-methyldeoxyguanosine (O^6-MedGuo), 7-MedGuo, O^4-methyldeoxythymidine (O^4-MedThd) and other adducts (Figure 2).

HPB-releasing DNA adducts were detected in human lung (Foiles *et al.*, 1991). Mean levels (fmol/mg DNA) were 11 ± 16 in nine smokers and 0.9 ± 2.3 in eight nonsmokers. Mean adduct levels in tracheobronchus were 16 ± 18 in four smokers and 0.9 ± 1.7 in four nonsmokers. In another study, HPB-releasing adducts were not detected in lung samples from four individuals, two of whom were confirmed smokers (detection limit reported as 8–50 fmol/mg DNA) (Blömeke *et al.*, 1996).

Methyl DNA adducts have been detected in tissues or cells of smokers and nonsmokers in several studies. Levels of 7-MedGuo were higher in pulmonary alveolar cells or bronchial tissues of smokers than nonsmokers (Mustonen *et al.*, 1993; Petruzzelli *et al.*, 1996) in two studies, while a third showed no difference (Kato *et al.*, 1995). Sample sizes and their origins were insufficient to judge the effects of smoking in four other studies (Wilson *et al.*, 1989; Shields *et al.*, 1990; Kato *et al.*, 1993; Blömeke *et al.*, 1996). Levels

of 7-alkylguanines in DNA from larynx tissue increased with the amount of smoking (Szyfter *et al.*, 1996). Levels of 7-MedGuo plus 7-(2-hydroxyethyldeoxyguanosine) were higher in lymphocytes of smokers compared with nonsmokers (Kumar & Hemminki, 1996). There was no effect of smoking or exposure to secondhand tobacco smoke on levels of O^6-MedGuo in human peripheral and cord blood DNA (Georgiadis *et al.*, 2000), and no difference in O^6-MedGuo levels in placenta from smokers and nonsmokers (Foiles *et al.*, 1988). Some of the methyl DNA adducts detected in these studies may have originated from NNK, but there are other sources of methyl DNA adducts in cigarette smoke, notably *N*-nitrosodimethylamine.

(iv) *Excretion*

Urine is the only route of excretion of NNK metabolites for which data from studies with humans are currently available (see Section on metabolism above and Table 12). Studies in laboratory animals indicate that urine is the major route of excretion of NNK metabolites (see Section 4.1.2(*d*)).

(*b*) N′-*Nitrosonornicotine (NNN)*

(i) *Absorption*

NNN has been detected in the saliva of snuff dippers, chewers of betel quid with tobacco, users of *khaini*, *gudhaku*, *toombak* and *mishri* and reverse smokers. The data are presented in Section 1.4.2(*a*).

Absorption of NNN has been demonstrated by detection of NNN and NNN-*N*-Gluc in the urine in smokeless tobacco users and smokers (Stepanov & Hecht, 2005; see Section 4.1.1(*b*)(iv)).

(ii) *Distribution*

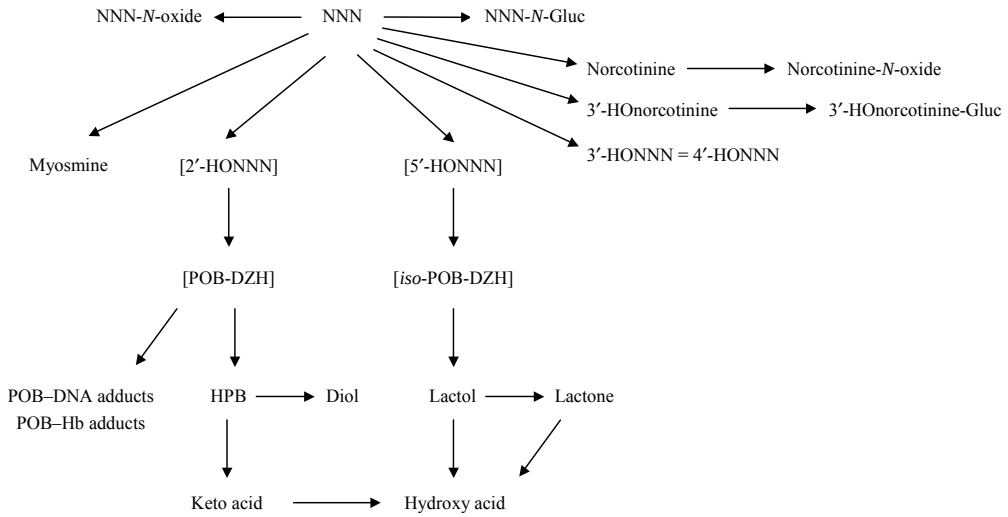
NNN was detected in two of 17 samples of pancreatic juice from smokers at levels of 68.1 and 242 ng/mL juice. It was not detected in the pancreatic juice from nine nonsmokers (Prokopczyk *et al.*, 2002). Pyridyloxobutyl–haemoglobin adducts have been detected in smokeless tobacco users and smokers, and pyridyloxobutyl–DNA adducts have been detected in smokers. These adducts could arise from NNN or NNK and are discussed in the section on NNK.

(iii) *Metabolism*

Introduction

The metabolic pathways of NNN and modes of DNA adduct formation are shown in Figure 6, and structures of the metabolites are illustrated in Figure 7. This information is based on studies *in vitro*, in laboratory animals and in humans. Specific pathways that have been observed in in-vitro studies with human tissues or enzymes or *in vivo* in humans are discussed below.

Figure 6. Metabolism of NNN and formation of adducts, based on studies in laboratory animals and humans

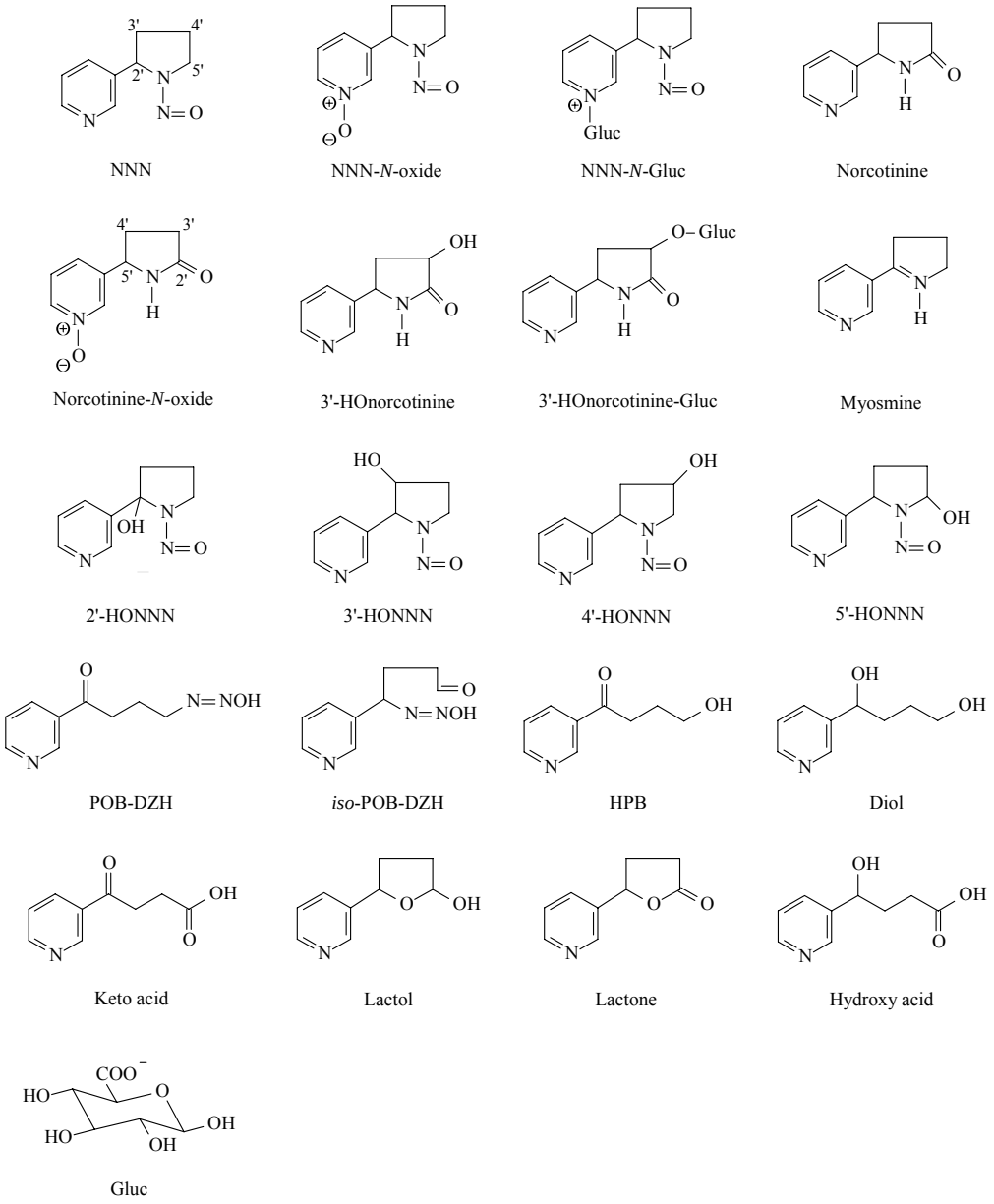


DZH, diazohydroxide; Gluc, glucuronide; Hb, haemoglobin; HO, hydroxy; HPB, 4-hydroxy-1-(3-pyridyl)-1-butanone or keto alcohol; NNN, *N*′-nitrosonornicotine; POB, 4-oxo-4-(3-pyridyl)-1-butyl or pyridyloxobutyl

NNN can undergo pyridine *N*-oxidation to yield NNN-*N*-oxide and pyridine *N*-glucuronidation to yield NNN-*N*-Gluc. Denitrosation and oxidation of NNN yields myosmine or norcotinine, and the latter can undergo pyridine *N*-oxidation to yield norcotinine-*N*-oxide. NNN also is metabolized to 3′-hydroxynorcotinine (3′-HOnorcotinine) but it is not clear whether this proceeds via norcotinine or by an independent pathway. 3′-HOnorcotinine undergoes glucuronidation on its hydroxyl group to yield 3′-HOnorcotinine-Gluc.

Hydroxylation of the pyrrolidine ring of NNN produces 2′-hydroxy-NNN (2′-HONNN), 3′-HONNN, 4′-HONNN and 5′-HONNN. While 3′-HONNN and 4′-HONNN are stable metabolites, 2′-HONNN and 5′-HONNN, which are α-hydroxynitrosamines, are unstable. 2′-HONNN spontaneously undergoes ring opening to yield POB-DZH. POB-DZH reacts with water to form HPB. POB-DZH also reacts with DNA and haemoglobin to form adducts that are believed to have structures identical to those formed by the POB-DZH pathway in NNK metabolism. HPB can be further oxidized to keto acid or reduced to diol. 5′-HONNN spontaneously undergoes ring opening to produce 1-(3-pyridyl)-4-oxo-1-butanediazohydroxide (*iso*-POB-DZH), which reacts with water to form 5-(3-pyridyl)-2-hydroxytetrahydrofuran (lactol). Lactol can be oxidized to 5-(3-pyridyl)tetrahydrofuran-2-one (lactone) or 4-hydroxy-4-(3-pyridyl)butanoic acid (hydroxy acid). Thus, HPB and keto acid are indicative of the metabolic activation of NNN by 2′-hydroxylation whereas the lactol and the hydroxy acid are indicative of the metabolic activation of NNN by 5′-hydroxylation.

Figure 7. Structures of NNN and metabolites



DZH, diazohydroxide; Gluc, glucuronide; HO, hydroxy; HPB, 4-hydroxy-1-(3-pyridyl)-1-butanone or keto alcohol; NNN, *N*′-nitrosonornicotine; POB, 4-oxo-4-(3-pyridyl)-1-butyl or pyridyloxobutyl

In-vitro studies in human tissues and cells

Metabolism of NNN by human tissues *in vitro* has been reviewed (Hecht, 1998). NNN-*N*-oxide and hydroxy acid (from 5′-hydroxylation) have been detected after incubation of cultured human tissues — buccal mucosa, trachea, oesophagus, bronchus, peripheral lung and urinary bladder — with NNN (Castonguay *et al.*, 1983c). Smaller amounts of keto acid (from 2′-hydroxylation) were also observed (Castonguay *et al.*, 1983c). Adult and fetal human oesophageal cultures metabolized NNN to hydroxy acid (5′-hydroxylation), keto acid (2′-hydroxylation) and NNN-*N*-oxide (Chakradeo *et al.*, 1995). Human oesophageal microsomes also metabolized NNN by 5′-hydroxylation and 2′-hydroxylation; and the latter was partially inhibited by troleandomycin, an inhibitor of CYP3A (Smith *et al.*, 1998).

Human liver microsomes metabolically activate NNN by 5′-hydroxylation to yield lactol and by 2′-hydroxylation to yield keto alcohol. Consistently, 5′-hydroxylation predominates (Hecht, 1998). Overall rates of metabolic activation of NNN were greater than those of NNK, NNAL or benzo[*a*]pyrene in human liver microsomes (Staretz *et al.*, 1997a). At concentrations of 0.8–1 μM NNN, metabolism by human liver microsomes was mainly by 5′-hydroxylation and the reaction was strongly correlated with coumarin 7-hydroxylation, which suggests the involvement of CYP2A6 (Patten *et al.*, 1997). 2′-Hydroxylation correlated with 6β-hydroxylation of testosterone, a CYP3A4-specific activity (Patten *et al.*, 1997).

CYPs have been identified as the major catalysts of NNN metabolic activation by α-hydroxylation. Human CYP2A6 metabolized NNN exclusively by 5′-hydroxylation, with a K_m of 2.1 μM (Patten *et al.*, 1997). The K_ms for 5′-hydroxylation of (*R*)-NNN and (*S*)-NNN were 22 μM and 2.3 μM, respectively (Wong *et al.*, 2005b). Rates of metabolism of NNN by CYP2E1 and 2D6 were much lower (Patten *et al.*, 1997). Metabolism of NNN by expressed human CYP3A4 was specific for 2′-hydroxylation (HPB formation) with a K_m of 304 μM (Patten *et al.*, 1997). Human CYP2A13 metabolized both (*R*)- and (*S*)-NNN by 5′-hydroxylation to lactol, with K_ms of 24 and 23 μM, respectively, and metabolized (*R*)-NNN to HPB with a K_m of 21 μM (Wong *et al.*, 2005b). Studies with *Salmonella typhimurium* strains that co-express human CYPs demonstrated that CYP2A6 was the most effective catalyst of NNN metabolic activation among 11 CYPs tested (Fujita & Kamataki, 2001).

In summary, studies with human tissues clearly demonstrate the metabolic activation of NNN; 5′-hydroxylation predominates in liver microsomes and both 2′-hydroxylation and 5′-hydroxylation are observed to comparable extents in oesophageal microsomes. NNN metabolism has also been observed in a variety of cultured tissues. Human CYP2A6 and CYP2A13 are effective catalysts of NNN α-hydroxylation.

In-vivo studies

NNN-*N*-Gluc has been detected in the urine of smokeless tobacco users and smokers (Stepanov & Hecht, 2005). Keto acid and the enantiomers of hydroxy acid have been quantified in the urine of smokers and abstinent smokers who used nicotine replacement therapy to test the hypothesis that (*S*)-hydroxy acid could be a biomarker of metabolic

activation of NNK and NNN while (*R*)-hydroxy acid would be formed predominantly from nicotine as indicated by studies with rats (Hecht *et al.*, 1999b; Trushin & Hecht, 1999). (*R*)-Hydroxy acid was the major enantiomer in human urine. The amount of (*S*)-hydroxy acid was far greater than that which could be formed from NNK and NNN. Both (*S*)-hydroxy acid and keto acid were formed in substantial amounts from nicotine, which precludes their use as biomarkers of NNN uptake in smokers.

HPB-releasing haemoglobin adducts and DNA adducts have been detected in tobacco users. These adducts can be formed either from NNK or from 2′-hydroxylation of NNN, and are discussed in section 4.1.1(*a*)(iii).

(iv) *Excretion*

NNN and NNN-*N*-Gluc have been detected in the urine of smokeless tobacco users and smokers (Stepanov & Hecht, 2005). The data are presented in Section 1.4.2(*b*).

(*c*) N′-*Nitrosoanabasine (NAB)*

(i) *Absorption*

NAB has been detected in the saliva of users of *toombak*, *khaini* tobacco and *gudhaku***.** The data are presented in Section 1.4.3(*a*).

Absorption of NAB by smokeless tobacco users and smokers has been demonstrated by detection of NAB and NAB-*N*-Gluc in urine (Stepanov & Hecht, 2005).

(ii) *Distribution*

No data were available to the Working Group

(iii) *Metabolism*

In experiments with 11 strains of *S. typhimurium* YG7108, each of which co-expresses a form of human CYPs, CYP 3A4, 2A6, 1A1 and 3A5 were capable of converting NAB to mutagenic products. CYP3A4 was the best catalyst (Fujita & Kamataki, 2001).

(iv) *Excretion*

NAB and NAB-*N*-Gluc have been detected in the urine of smokers and smokeless tobacco users (Stepanov & Hecht, 2005). The data are presented in Section 1.4.3(*b*).

(*d*) N′-*Nitrosoanatabine (NAT)*

(i) *Absorption*

NAB has been detected in the saliva of users of *toombak*, *khaini* and *gudhaku*. The data are presented in Section 1.4.4(*a*).

Absorption of NAT has been demonstrated by the detection of NAT and NAT-*N*-Gluc in the urine of smokeless tobacco users and smokers (Stepanov & Hecht, 2005).

(ii) *Distribution*

No data were available to the Working Group.

(iii) *Metabolism*

In experiments with 11 strains of *S. typhimurium* YG7108, each of which co-expresses a form of human CYPs, CYP2A6 was capable of converting NAT to mutagenic products (Fujita & Kamataki, 2001).

(iv) *Excretion*

NAT and NAT-*N*-Gluc have been detected in the urine of smokers and smokeless tobacco users (Stepanov & Hecht, 2005). The data are presented in Section 1.4.4(*b*).

4.1.2 *Experimental systems*

(*a*) *NNK and NNAL*

Studies on the absorption, distribution, metabolism and excretion of NNK in experimental systems have been comprehensively reviewed (Hecht, 1998). The reader is referred to that review for detailed coverage of the literature. Selected studies from the previous review that illustrate important points as well as more recent studies pertinent to the evaluation are presented below.

(i) *Absorption*

Beagle dogs were exposed to a single spray bolus of dissolved NNK in the distal trachea (0.48 nmol/dog) (Gerde *et al.*, 1998). NNK was rapidly absorbed and extensively metabolized in the tracheal mucosa. Most NNK appeared rapidly in the blood that drains the airway mucosa, but a phase of slow clearance was also observed. During absorption, NNK was distributed within the entire depth of the mucosa to the tracheal cartilage. A portion was bound to the mucin component of the mucous lining. First-pass metabolism and activation of NNK in the airway mucosa were sufficiently rapid to cause levels of binding at the site of absorption that were 20-fold those at distal tissues, which indicates a mechanism by which NNK could act as a carcinogen at the site of entry. In a comparison of NNK and benzo[*a*]pyrene, it was concluded that NNK is diffused into the mucosa sufficiently rapidly for blood perfusion to limit clearance, whereas benzo[*a*]pyrene is diffused into the mucosa more slowly. NNK passes into the blood about 100 times more rapidly than benzo[*a*]pyrene. NNK and its metabolites swiftly disperse thoughout the mucosa, whereas benzo[*a*]pyrene and its activated metabolites are confined to the target epithelium. NNK was metabolized in the airway mucosa at least 15 times faster than benzo[*a*]-pyrene, but NNK metabolites bound less effectively than those of benzo[*a*]pyrene at the site of entry. The rapid absorption of NNK at the site of entry could lead to accumulation at peripheral sites in the lung, which is consistent with its ability to induce adenocarcinoma (Gerde *et al.*, 1998). Rapid absorption of NNK has also been observed after admi-

nistration to rodents and monkeys by various routes (Castonguay *et al.*, 1983b; Tjälve & Castonguay, 1983; Castonguay *et al.*, 1985a; Tjälve, 1991).

(ii) *Distribution*

Audoradiographic studies demonstrate that, 1 min after intravenous administration of [carbonyl-^{14}C]NNK to rats, radioactivity was homogeneously distributed in most tissues of the body at a level similar to that in the blood (Castonguay *et al.*, 1983b). At later time intervals, accumulation of bound radioactivity was observed in tissues such as the lung and nasal mucosa, which are targets of NNK carcinogenicity. Relatively large amounts of unbound radioactivity are also observed in the stomach contents and melanin-containing tissues, due to the basicity of NNK and NNAL (Castonguay *et al.*, 1983b; Tjälve & Castonguay, 1983; Castonguay *et al.*, 1985a; Hecht, 1998). These studies show that NNK is distributed rapidly and homogeneously throughout the body and has the ability to cross cellular membranes freely and partition evenly in the intra- and extracellular tissue water (Castonguay *et al.*, 1983b). Initially, strong labelling was observed in parts of the nasal mucosa, in the liver, bronchial mucosa, adrenal cortex, preputial gland, salivary gland and stomach contents. At later time points, radioactivity persisted in certain tissues and was seen in the kidney, urinary bladder and gastrointestinal contents (Castonguay *et al.*, 1983b). Similar results have been obtained in autoradiographic and other studies carried out in Syrian golden hamsters, dogs and marmoset monkeys by various routes of administration (Tjälve & Castonguay, 1983; Castonguay *et al.*, 1985a; Gerde *et al.*, 1998; Tjälve, 1991).

Micro-autoradiographic studies in Fischer 344 rats injected with [CH_3-^{3}H]NNK showed that the highest degree of labelling in the lung was in the Clara cells (Belinsky *et al.*, 1987a); in the nasal passages, the highest degree of labelling was in Bowman's glands, Steno's gland and serous glands of the respiratory mucosa, with a lower degree of labelling in the respiratory and olfactory epithelia (Belinsky *et al.*, 1987b). Similar results were obtained with [carbonyl-^{14}C]NNK (Tjälve *et al.*, 1985; Tjälve, 1991).

Pharmacokinetic studies of NNK and NNAL in rats demonstrated large volumes of distribution of NNK (321 ± 137 mL) and NNAL (2772 ± 1423 mL) (Wu *et al.*, 2002). The enantiomers of NNAL appear to be distributed differently in the body, as indicated by the apparent volumes of distribution: 1792 ± 570 mL for (*S*)-NNAL and 645 ± 230 mL for (*R*)-NNAL, a difference that was significant (Zimmerman *et al.*, 2004). These data suggest extensive tissue binding that is greater for (*S*)-NNAL. Tissue distribution studies demonstrated that (*S*)-NNAL was retained in the lung 24 h after administration; the (*S*):(*R*) ratio increased from 1.1 1 h after administration to 4.23 24 h after administration (Zimmerman *et al.*, 2004). An increase of this magnitude was observed only in the lung, which suggests that (*S*)-NNAL is stereoselectively retained in the rat lung, possibly at a receptor site. This tissue distribution of NNAL may partially explain the initial accumulation of radioactivity in certain rat tissues, as seen by autoradiography, as well as the relatively long retention of (*S*)-NNAL seen in smokers and smokeless tobacco users.

(iii) *Metabolism*

Extensive studies of the metabolism of NNK have been carried out *in vitro* and *in vivo* in a variety of species including rats, hamsters, mice, rabbits, pigs and monkeys (reviewed in Hecht, 1998). Figure 2 summarizes the metabolism of NNK determined from these investigations. Virtually all systems examined conform to this general scheme, with major pathways of metabolism generally being reduction to NNAL and α-hydroxylation of NNK and NNAL. In the conversion of NNK to NNAL, (*S*)-NNAL is the predominant enantiomer formed in rat and mouse liver and lung microsomes and cytosol, as well as red blood cells (Upadhyaya *et al.*, 2000).

In-vitro studies of NNK metabolism

In-vitro studies of NNK metabolism have been comprehensively reviewed (Hecht, 1998). Studies of the kinetic parameters for NNK metabolism mediated by microsomal preparations from tissues of laboratory animals (Table 13) and relevant CYP enzymes (Table 11) are discussed below. Other selected studies of NNK metabolism *in vitro* are also included.

Monkey

Kinetic parameters for NNK metabolism have been reported for patas monkey lung and liver microsomes (Table 13; Smith *et al.*, 1997). In the lung, HPB is formed with the greatest efficiency, followed by NNK-*N*-oxide, the keto aldehyde and NNAL (Table 13; Smith *et al.*, 1997). K_m values for keto aldehyde, HPB and NNK-*N*-oxide formation were about 5–10 μM. The K_m for NNAL formation was much higher, however, which is consistent with the hypothesis that this metabolite is not formed by the same enzymes that catalyse α-hydroxylation (Maser *et al.*, 1996; Maser, 1998; Maser *et al.*, 2000; Finckh *et al.*, 2001). Similar results were obtained in patas monkey liver microsomes (Table 13). The kinetic parameters for NNK metabolism by expressed monkey CYPs have not been reported, but antibody and chemical inhibition studies imply that members of the CYP1A and 2A subfamilies are important catalysts of NNK metabolism (Smith *et al.*, 1997). The metabolism of NNK was also studied in short-term cultures of patas and cynomolgus monkey lung, and in Fischer 344 rat lung for comparison. Substantial amounts of metabolites from the α-hydroxylation pathway of metabolic activation were observed, together with the formation of NNK-*N*-oxide and NNAL. The metabolism of NNK by cultured monkey lung was generally similar to that observed in rat lung, which indicates that there are no major species differences between rodents and non-human primates in the pulmonary metabolism of NNK (Hecht *et al.*, 2000).

Rabbit

NNK metabolism by rabbit nasal, but not liver or lung, microsomes has been studied; kinetic parameters have not been reported (Hong *et al.*, 1992; Hecht, 1998). Kinetic parameters for CYPs purified from rabbit nasal microsomes — CYP2A10/2A11 (a mixture of two CYPs previously referred to as NMa) and CYP2G1 (previously NMb) — have been

Table 13. Apparent steady-state kinetic parameters for microsome-mediated NNK metabolism

Species/tissue	Metabolite	Kinetic parameters			Experimental conditions	Reference
		V_{max}[a]	K_m (μM)	V_{max}/K_m[b]		
Female Patas monkey lung	Keto aldehyde	5.3 ± 0.4	10.3 ± 0.8	0.51 ± 0.06	1–20 μM NNK; 30-min incubation, 0.5 mg/mL microsomal protein.	Smith *et al.* (1997)
	HPB	19.1 ± 0.8	4.9 ± 0.2	3.9 ± 0.2		
	NNK-*N*-oxide	11.0 ± 0.3	5.4 ± 0.2	2.0 ± 0.01		
	NNAL[c]	479 ± 35	902 ± 21	0.53 ± 0.04		
Female Patas monkey liver	Keto aldehyde	37.7 ± 1.9	8.2 ± 0.3	4.6 ± 0.3	1–50 μM NNK; 20-min incubation, 0.25 mg/mL microsomal protein.	
	HPB	37.4 ± 1.0	8.1 ± 0.2	4.6 ± 0.2		
	NNAL[c]	3470 ± 103	474 ± 28	7.3 ± 0.5		
Male Sprague-Dawley rat lung	Keto aldehyde	11.7 ± 1	28.9 ± 1.2	0.40 ± 0.04	1–50 μM NNK, 30-min incubation, 0.25 mg/mL microsomal protein.	Smith *et al.* (1992a)
	HPB	14.6 ± 1.0	7.0 ± 0.5	2.1 ± 0.2		
	NNK-*N*-oxide	35.1 ± 2.3	10.4 ± 0.9	3.4 ± 0.4		
	NNAL	195.3 ± 9	178 ± 10	1.1 ± 0.1		
Male Fischer 344 rat liver	Formaldehyde	1478	5	296	12.5–4000 μM NNK, 0.55 mg/mL microsomal protein.	Castonguay *et al.* (1991)
	Formaldehyde	197	534	0.37		
Male Sprague-Dawley rat liver	Keto aldehyde	153 ± 16	234 ± 38	0.65 ± 0.13	5–200 μM NNK, 5-min incubation, 0.75 mg/mL microsomal protein.	Guo *et al.* (1992)
	HPB	156 ± 8	211 ± 20	0.74 ± 0.08		
	Keto aldehyde	381 ± 51	149 ± 32	2.6 ± 0.6	As above, but animals were treated with 3-methylcholanthrene	
	HPB	270 ± 43	246 ± 54	1 ± 0.3		
	Keto aldehyde	329 ± 35	119 ± 22	2.8 ± 0.6	As above, but animals were treated with phenobarbital.	
	HPB	358 ± 27	177 ± 20	2.0 ± 0.3		
	NNK-*N*-oxide	140 ± 10	57 ± 9	2.5 ± 0.4		
	Keto aldehyde	550 ± 44	133 ± 21	4.1 ± 0.7	As above, but animals were treated with pregnenolone 16α-carbonitrile.	
	HPB	247 ± 18	187 ± 24	1.3 ± 0.2		
	NNK-*N*-oxide	167 ± 15	103 ± 21	1.6 ± 0.4		
Male Sprague-Dawley rat nasal mucosa	Keto aldehyde	2833 ± 81	9.6 ± 0.3	295 ± 12	1–100 μM NNK, 10-min incubation, 0.013 mg/mL microsomal protein.	Smith *et al.* (1992a)
	HPB	3275 ± 60	10.1 ± 0.2	324 ± 9		

Table 13 (contd)

Species/tissue	Metabolite	Kinetic parameters			Experimental conditions	Reference
		V_{max}[a]	K_m (μM)	V_{max}/K_m[b]		
Female A/J mouse lung	Formaldehyde	57.2 ± 2.2	5.6 ± 0.9	10.2 ± 1.7	1–20 μM NNK, 30-min incubation, 0.25 mg/mL microsomal protein.	Smith *et al.* (1990)
	HPB	56.0 ± 3.8	5.6 ± 0.9	10.0 ± 1.7		
	Keto acid	4.2 ± 0.5	9.2 ± 1.0	0.456 ± 0.074		
	NNK-*N*-oxide	54.2 ± 1.3	4.7 ± 0.9	11.5 ± 2.2		
	NNAL[c]	1322 ± 10	2541 ± 15	0.52 ± 0.005		
	Keto aldehyde	58.9 ± 2.6	23.7 ± 2.6	2.5 ± 0.3	0.5–100 μM NNK, 30-min incubation, 0.25 mg/mL microsomal protein.	Peterson *et al.* (1991a)
	HPB	32.5 ± 2.5	3.6 ± 0.9	9.0 ± 2.4		
Female A/J mouse lung	Keto aldehyde	34.0	4.9	6.9	1–10 μM NNK, 30-min incubation, 0.25 mg/mL microsomal protein.	Smith *et al.* (1993)
	HPB	38.1	2.6	15		
	NNK-*N*-oxide	60.0	1.8	33		
	Keto aldehyde	31.8	5.0	6.4	As above, but animals were treated with PEITC (1 μmol/g diet).	
	HPB	35.1	2.9	12		
	NNK-*N*-oxide	51.0	1.8	28		
	Keto aldehyde	25.7	4.7	5.5	As above, but animals were treated with PEITC (3 μmol/g diet).	
	HPB	23.0	2.4	9.6		
	NNK-*N*-oxide	36.7	1.6	23		
	Keto aldehyde	84.7 ± 2.8	4.5 ± 0.4	19 ± 2	0.25–20 μM NNK, 30-min incubation, 0.25 mg/mL microsomal protein.	
	HPB	62.8 ± 1.3	1.9 ± 0.2	33 ± 4		
	NNK-*N*-oxide	83.3 ± 3.6	2.0 ± 0.3	42 ± 7		
	Keto aldehyde[d]	89.2 ± 6.3	24.0 ± 2.7	3.7 ± 0.5	As above, but with PEITC (400 nM) added to the incubation mixtures.	Smith *et al.* (1993)
	HPB[d]	60.4 ± 1.8	14.9 ± 0.8	4.1 ± 0.2		
	NNK-*N*-oxide[d]	85.8 ± 4.6	17.9 ± 1.7	4.8 ± 0.5		
	Keto aldehyde	71 ± 3	4.8 ± 0.7	15 ± 2	0.25–50 μM NNK, 15-min. incubation, 0.25 mg/mL microsomal protein.	Jalas *et al.* (2003b)
	HPB	93 ± 3	3.0 ± 0.4	31 ± 4		
	NNK-*N*-oxide	109 ± 4	2.1 ± 0.3	52 ± 8		

Table 13 (contd)

Species/tissue	Metabolite	Kinetic parameters			Experimental conditions	Reference
		V_{max}[a]	K_m (μM)	V_{max}/K_m[b]		
Female A/J mouse liver	Keto aldehyde	245 ± 17	24 ± 4	10 ± 2	0.5–100 μM NNK, 15-min incubation, 0.25 mg/mL microsomal protein.	Nunes *et al.* (1998)
	HPB	100 ± 7	18 ± 4	5.6 ± 1.3		
	Keto aldehyde[d]	213 ± 41	23 ± 12	9.3 ± 5.2	As above, but with 2.5 μM 4-HPO added	
	HPB[d]	77 ± 13	17 ± 8	4.5 ± 2.3		
	Keto aldehyde[d]	210 ± 16	24 ± 4	8.8 ± 1.6	As above, but with 5.0 μM 4-HPO added	
	HPB[d]	69 ± 11	17 ± 8	4.1 ± 2.0		
	Keto aldehyde[d]	170 ± 14	22 ± 5	7.7 ± 1.9	As above, but with 10 μM 4-HPO added	
	HPB[d]	71 ± 4	18 ± 3	3.9 ± 0.7		
	Keto aldehyde[d]	78 ± 1	22 ± 8	3.5 ± 1.3	As above, but with 20 μM 4-HPO added	
	HPB[d]	44 ± 5	18 ± 5	2.4 ± 0.7		
	Keto aldehyde	173 ± 6	19.1 ± 2	9.1 ± 0.7	1–100 μM NNK, 10-min incubation, 0.25 mg/mL microsomal protein.	Peterson *et al.* (1991a)
	HPB	239 ± 11	73.8 ± 6.8	3.2 ± 0.3		
	Keto aldehyde	132 ± 11	5.5 ± 0.3	24 ± 2	1–10 μM NNK, 10-min incubation, 0.50 mg/mL microsomal protein.	Smith *et al.* (1993)
	HPB	60.4 ± 3.1	5.1 ± 0.2	11.8 ± 0.8		
	NNK-*N*-oxide	8.0 ± 0.6	8.8 ± 0.5	0.91 ± 0.09		
	Keto aldehyde	77.0 ± 9.3	5.4 ± 0.4	14.3 ± 2.0	As above, but animals were treated with PEITC (3 μmol/g diet).	
	HPB	39.3 ± 2.5	5.3 ± 0.3	7.4 ± 0.6		
	NNK-*N*-oxide	5.6 ± 0.7	9.1 ± 0.3	0.62 ± 0.08		

Adapted from Jalas *et al.* (2005)

HPB, 4-hydroxy-1-(3-pyridyl)-1-butanone; 4-HPO, 4-hydroxy-1-phenyl-1-octanone; K_m, Michaelis constant; NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; NNK, 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone; PEITC, phenethyl isothiocyanate; V_{max}, maximum velocity

[a] Units are pmol/mg/ min.

[b] Units are pmol/mg/min/μM.

[c] NNK concentrations up to 1000 μM were used to determine kinetic parameters for NNAL formation.

[d] Values are V_{max}', K_m', and V_{max}'/K_m', respectively, due to the presence of an inhibitor in the incubation mixtures.

determined (Hong *et al.*, 1992). In the absence of exogenous cytochrome b_5, reconstituted CYPs 2A10/2A11 exhibited K_m values for NNK α-hydroxylation that were similar to those for human CYP2A13 (Table 12; Hong *et al*., 1992; Su *et al*., 2000; Zhang *et al*., 2002). CYPs 2A10/2A11 exhibited lower K_m and higher V_{max}/K_m values for the keto aldehyde formation than those determined using CYP2G1 (Table 11; Hong *et al.*, 1992). Nicotine was a competitive inhibitor of CYP2A10/2A11-mediated NNK metabolism (Table 11; Hong *et al.*, 1992).

Rat

Studies in isolated perfused liver and lung demonstrated that, at a concentration of 35 nM, NNK was rapidly eliminated (Schrader *et al.*, 1998). The clearance was almost exclusively mediated by metabolism. The kinetics of NNK metabolism in the liver was substantially faster than that in the lung but, on a per gram basis, lung clearance was faster. Products of α-hydroxylation were the major metabolites in the liver and NNK-*N*-oxide was the major metabolite in the lung, followed by α-hydroxylation products. Studies in rat lung and liver cells gave similar results and indicated a correlation between metabolite profiles in lung cells and urinary metabolites obtained after treatment of rats with NNK and modifiers (Schneider *et al.*, 1999). A study of NNK metabolism in rat alveolar type II cells demonstrated substantial metabolism by α-hydroxylation at low concentrations of NNK (50 nM), a concentration probably relevant to human exposure (Schrader *et al.*, 2000).

The kinetics of NNK metabolism have been studied extensively using microsomes prepared from Sprague-Dawley and Fischer rat lung, liver and nasal mucosa (Table 13). In rat lung, NNK-*N*-oxide is the major NNK metabolite, followed by HPB, NNAL and the keto aldehyde (Hecht, 1998). K_m values for HPB and NNK-*N*-oxide formation were similar to those in patas monkey lung (Table 13). The kinetic parameters of NNK metabolism in rat liver and the effects of various inducers of CYP on these parameters have been evaluated in several studies. The K_m values for the keto aldehyde and HPB formation by rat liver microsomes were higher than those in human or patas monkey liver microsomes (Table 13; Guo *et al*., 1992; Patten *et al*., 1996; Smith *et al*., 1997). Treatment of rats with phenobarbital, which induces expression of CYP2B enzymes (Soucek & Gut, 1992; Whitlock & Denison, 1995), led to detectable levels of NNK-*N*-oxide and slightly increased the efficiency of POB and HPB formation (Table 13; Guo *et al.*, 1992). Treatment of rats with pregnenolone 16α-carbonitrile, an inducer of CYP3A enzymes (Heuman *et al.*, 1982; Soucek & Gut, 1992; Whitlock & Denison, 1995), also led to enhanced efficiency of keto aldehyde and HPB formation, as well as to the formation of NNK-*N*-oxide (Table 13; Guo *et al.*, 1992).

Microsomes prepared from rat nasal mucosa are better catalysts of NNK bioactivation than any other microsomal system investigated to date (Table 13). The K_m values for HPB formation were comparable in rat lung and nasal mucosa, whereas the K_m for the keto aldehyde formation by rat nasal mucosal microsomes was threefold lower than that in lung microsomes (Table 13). The much higher catalytic efficiency of NNK metabolism in rat nasal mucosal microsomes than lung or liver was clearly due to the larger V_{max} values in

the nasal mucosa (Table 13). These data are consistent with a role for CYP2A3 (discussed below) as an important catalyst of NNK α-hydroxylation in lung and nasal mucosa.

The kinetic parameters for NNK metabolism by rat CYPs have been studied only for two enzymes — CYP2A3 and 2B1 (Table 12; Guo *et al.*, 1991a; Jalas *et al.*, 2003a). CYP2A3 is expressed in both the nasal mucosa and lung, but not the liver (Su *et al.*, 1996). Both protein and mRNA levels are much greater in the nasal mucosa than in the lung (Su *et al.*, 1996; Gopalakrishnan *et al.*, 1999). CYP2A3 catalyses α-hydroxylation of NNK much more efficiently than CYP2B1. A comparison of the primary sequence of rat and human CYP2A enzymes showed that rat CYP2A3 was 85 and 87% identical to human CYPs 2A6 and 2A13, respectively. CYP2A3 catalyses NNK metabolism with an efficiency similar to that of human CYP2A13 (Honkakoski & Negishi, 1997; Jalas *et al.*, 2003a). The high catalytic efficiency of CYP2A3 for NNK α-hydroxylation most probably plays a role in the carcinogenicity of NNK to the rat lung and nasal mucosa (Jalas *et al.*, 2005).

CYP2B1 also catalyses the α-hydroxylation of NNK, but with much higher K_m and lower V_{max} values than CYP2A3 (Table 11; Guo *et al.*, 1991a). CYP2B1, unlike CYP2A3, also catalyses NNK-*N*-oxide formation (Table 11; Guo *et al.*, 1991a). Rat CYP2B1 and CYP2C6, together with human CYP2C8, are the only CYP enzymes reported to catalyse the *N*-oxidation of NNK (Guo *et al.*, 1991a; Smith *et al.*, 1992a; Lacroix *et al.*, 1993; Jalas *et al.*, 2005). Because NNK-*N*-oxide formation represents quantitatively the major CYP-catalysed pathway of NNK metabolism in the rat lung, studies have examined the enzyme(s) responsible for this reaction (Smith *et al.*, 1992b). Anti-2B1 antibodies do not inhibit the formation of this metabolite by rat lung microsomes (Smith *et al.*, 1992b). Furthermore, phenobarbital treatment of rats induced the formation of NNK-*N*-oxide catalysed by rat liver microsomes (Table 13), but did not significantly enhance the rate of formation of this metabolite catalysed by rat lung microsomes (Guo *et al.*, 1992).

Kinetic parameters for NNK metabolism for rat CYPs 1A1, 1A2, and 2C6 are also presented in Table 11 (Jalas *et al.*, 2005). Rat CYP1A1, similarly to human CYP1A1 (with which it shares 78% primary sequence identity; Soucek & Gut, 1992) preferentially catalyses keto aldehyde formation, but with much lower K_m values (Table 11; Smith *et al.*, 1995; Jalas *et al.*, 2005). The V_{max}/K_m values for keto aldehyde formation were an order of magnitude higher for the rat enzyme compared with the human enzyme, but these values were similar for HPB formation (Table 11; Smith *et al.*, 1995; Jalas *et al.*, 2005). In contrast to rat CYP1A1, rat CYP1A2 does not exhibit the regioselectivity observed with the orthologous human enzyme (primary sequence identity, 70%; Soucek & Gut, 1992) (Table 11; Smith *et al.*, 1992a, 1996; Jalas *et al.*, 2005).

Rat CYP2C6, similarly to rat CYP2B1 and human CYP2C8, catalyses the *N*-oxidation in addition to α-hydroxylation of NNK (Table 11; Guo *et al.*, 1991a; Smith *et al.*, 1992b; Lacroix *et al.*, 1993; Jalas *et al.*, 2005). The K_m values for CYP2C6 were much higher than those for CYP2B1, but higher V_{max} values led to V_{max}/K_m values that were similar between the two enzymes (except in the case of HPB formation) (Table 11; Guo *et al.*, 1991a; Jalas *et al.*, 2005). Rat CYP2C6 catalyses HPB formation about 10-fold more efficiently than

CYP2B1 (Table 11; Guo *et al.*, 1991a; Jalas *et al.*, 2005). Rat CYPs 2D1, 2D2, 3A1 and 3A2 do not metabolize NNK (Jalas *et al.*, 2005).

Mouse

Lung microsomes from female A/J mice have been used by several laboratories to determine the kinetic parameters of NNK metabolism (Table 13). The reported K_m values were consistently in the 1–10 μM range for keto aldehyde, HPB and NNK-*N*-oxide (Smith *et al.*, 1990; Peterson *et al.*, 1991a; Smith *et al.*, 1993; Jalas *et al.*, 2003b). Similarly to results with rat lung microsomes, NNK-*N*-oxide was the major metabolite (Table 13). In female A/J mouse liver microsomes, NNK was converted to keto aldehyde, HPB and NNK-*N*-oxide (Table 13). The K_m values were similar in mouse lung and liver microsomes, but the V_{max} values were generally higher for liver microsome-mediated formation of keto aldehyde and HPB (Table 13).

Kinetic parameters for NNK metabolism have been determined for mouse CYPs 2A4 and 2A5 (Felicia *et al.*, 2000; Jalas *et al.*, 2003b). CYP2A4 and CYP2A5 are more than 90% identical to human CYPs 2A6 and 2A13 and are expressed in many mouse tissues, including the liver and lung (Honkakoski & Negishi, 1997). These two mouse CYP2A enzymes differ in primary sequence by only 11 amino acids (of 494), but exhibit strikingly different substrate specificities (Lindberg & Negishi, 1989; Honkakoski & Negishi, 1997). Mutation of only one amino acid residue (Phe 209) in CYP2A5 to the corresponding residue in CYP2A4 (Leu 209) is sufficient to convert the substrate specificity of CYP2A5 to that of a 2A4-like enzyme (Lindberg & Negishi, 1989). Investigation of the kinetic parameters of NNK metabolism by these two highly similar CYPs revealed significant differences in their ability to catalyse NNK α-hydroxylation (Table 11; Felicia *et al.*, 2000). CYP2A5 exhibits a much lower K_m value and preferentially hydroxylates the α-methyl carbon of NNK, whereas CYP2A4 exhibits a much higher K_m value and preferentially catalyses the α-methylene hydroxylation of NNK (Table 11; Felicia *et al.*, 2000; Jalas *et al.*, 2003b). The K_m value for CYP2A5-mediated α-methyl hydroxylation of NNK is the lowest among those of the CYPs listed in Table 11.

Hamster

Kinetic parameters for NNK metabolism were determined in tissue slices from the lung, liver and kidney of female Syrian golden hamsters (Richter *et al.*, 2000). High and low K_m and V_{max} values were observed in the lung and liver for the formation of most NNK metabolites including hydroxy acid, keto acid, HPB, diol, NNK-*N*-oxide, NNAL-*N*-oxide and NNAL. K_m values ranged from 0.04 to 1952 μM. In the lung, α-hydroxylation accounted for 13–31% of metabolism. The liver showed the highest capacity for NNK metabolism, and α-hydroxylation accounted for 12–25% of the metabolites. The kidney showed a low capacity for NNK metabolism with α-hydroxylation accounting for < 3% of the metabolites. Conversion of NNK to NNAL was greatest in the kidney, followed by the liver and lung.

Summary

In the species that have been studied, a number of CYP2A enzymes are excellent catalysts of NNK α-hydroxylation, but more research is needed to clarify the contribution of individual CYPs to microsome-mediated NNK metabolism in animals. The K_m values for NNK α-hydroxylation in patas monkey lung and liver microsomes are consistent with the involvement of a CYP2A13 orthologue, but further study is required. The K_m values observed using rat lung microsomes are consistent with the involvement of CYP2A3 which is closely related to human CYP2A13 or mouse CYP2A5. The rat liver does not express a CYP2A6 or 2A13 orthologue (Honkakoski & Negishi, 1997; Gopalakrishnan *et al.*, 1999) and the K_m values for NNK metabolism by liver microsomes are consistent with the involvement of CYP2B1. Kinetic data on NNK metabolism by other rat CYPs is needed to assess better the involvement of individual enzymes in this species. The K_m values for NNK metabolism in mouse lung and liver microsomes are similar to those determined for CYP2A5. It seems probable that CYP2A5 plays a role in NNK bioactivation in mice; however, additional studies are needed to define better the contribution of CYP2A5 in both the lung and liver. Table 14 summarizes K_m values and regioselectivity in NNK metabolism for both laboratory animal and human CYPs.

In-vitro studies of NNAL metabolism

Rat liver microsomes convert NNAL to lactol and hydroxy acid (products of α-methylene hydroxylation), to diol and pyridylTHF (products of α-methyl hydroxylation), as well as to NNAL-*N*-oxide, $NNAL(ADP)^+$ and NNK (Peterson *et al.*, 1994; Staretz *et al.*, 1997b; Upadhyaya *et al.*, 2000). In rat pancreatic microsomes, only $NNAL(ADP)^+$ was observed (Peterson *et al.*, 1994). Rat liver microsomes and co-factors convert NNAL predominantly to (*R*)-NNAL-*O*-Gluc; the uridine diphospho (UDP)-glucuronosyl transferase, UGT2B1, is an important catalyst of this reaction (Ren *et al.*, 1999).

Kinetic parameters for NNAL metabolism catalysed by microsomes have only been reported for A/J mouse lung microsomes (Table 15). K_m values for NNAL and NNK metabolism were similar (Tables 13 and 15), but V_{max} values for NNAL were almost an order of magnitude lower. Thus, *in vivo*, metabolic activation of NNAL may be a less important source of DNA-reactive electrophiles than metabolic activation of NNK.

NNAL-*N*-oxide was the major metabolite formed from (±)-, (*R*)- and (*S*)-NNAL in mouse lung microsomes (Table 15; Jalas & Hecht, 2003). NNAL-*N*-oxide was formed from (*S*)-NNAL at much greater maximal rates than from the other two substrates, but the K_m value was also higher for (*S*)-NNAL; this resulted in relatively similar V_{max}/K_m values for NNAL-*N*-oxide formation among all three substrates (Table 15; Jalas & Hecht, 2003).

Kinetic parameters for NNAL metabolism have also been determined with hamster liver, lung and kidney tissue slices (Richter *et al.*, 2000). NNAL was converted to hydroxy acid, NNAL-*N*-oxide and NNK in all three tissue preparations, as well as to NNK-*N*-oxide in the liver and to diol in the lung (Richter *et al.*, 2000). K_m values for these metabolites ranged from 1.6 μM for NNK formation in the lung to 1624 μM for NNAL-*N*-oxide formation in the same tissue (Richter *et al.*, 2000).

Table 14. Summary of K_m values and regioselectivity for cytochrome P450-mediated NNK metabolism

Metabolic pathway	Low K_m (< 50 μM)	Intermediate K_m (50–500 μM)	High K_m (> 500 μM)	Regioselectivity
α-Methylene hydroxylation	Human 2A13 Rabbit 2A10/2A11 Rat 2A3 Mouse 2A5	Human 2A6 Rabbit 2G1 Rat 1A1 Rat 1A2 Rat 2B1 Mouse 2A4	Human 1A1 Human 1A2 Human 2D6 Human 2E1 Human 3A4 Rat 2C6	α-Methylene > α-methyl Human 1A1 Human 2A13 Human 3A4 Rabbit 2G1 Rat 1A1
α-Methyl hydroxylation	Human 2B6 Human 2A13 Rabbit 2A10/2A11 Rat 2A3 Mouse 2A5	Human 1A1 Human 1A2 Human 2A6 Rat 1A1 Rat 1A2 Rat 2B1 Mouse 2A4	Human 2D6 Human 2E1 Human 3A4 Rat 2C6	α-Methylene ≅ α-methyl Human 2A6 Rabbit 2A10/2A11 Rat 1A2 Rat 2A3
N-Oxidation		Rat 2B1	Rat 2C6	α-Methylene < α-methyl Human 1A2 Human 2B6 Human 2D6 Human 2E1 Rat 2B1 Rat 2C6 Mouse 2A5

Adapted from Jalas *et al.* (2005)
K_m, Michaelis constant

Table 15. Apparent steady-state kinetic parameters for A/J mouse lung microsome-mediated metabolism of NNAL

Substrate	Metabolite	Kinetic parameters			Experimental conditions	Reference
		V_{max} [a]	K_m (μM)	V_{max}/K_m [b]		
(±)-NNAL	Lactol	6.6 ± 0.3	5.7 ± 0.9	1.2 ± 0.2	0.25–50 μM NNAL; 45-min incubation; 0.3 mg/mL microsomal protein.	Jalas & Hecht (2003)
	Diol	6.0 ± 0.3	3.7 ± 0.7	1.6 ± 0.3		
	NNAL-*N*-oxide	20 ± 0.4	2.2 ± 0.2	9.1 ± 0.8		
(*R*)-NNAL	Lactol	6.5 ± 0.4	5.7 ± 1	1.1 ± 0.2		
	Diol	5.8 ± 0.3	5.0 ± 0.9	1.2 ± 0.2		
	NNAL-*N*-oxide	13 ± 0.4	2.0 ± 0.2	6.5 ± 0.7		
(*S*)-NNAL	Lactol	14 ± 0.8	9.9 ± 1.5	1.4 ± 0.2		
	Diol	15 ± 0.6	7.8 ± 1	1.9 ± 0.3		
	NNAL-*N*-oxide	103 ± 2	9.4 ± 0.5	11 ± 0.6		

K_m, Michaelis constant; NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; V_{max}, maximum velocity
[a] Units are pmol/mg/min.
[b] Units are pmol/mg/min/μM.

The kinetic parameters for CYP-mediated NNAL metabolism have been reported only for three enzymes — mouse CYP2A5, rat CYP2A3 and human CYP2A13 (Table 16; Jalas & Hecht, 2003; Jalas *et al.*, 2003a). Similarly to results with mouse lung microsomes, the K_m values for CYP2A5-mediated NNAL metabolism were similar to those for CYP2A5-mediated NNK metabolism and were largely independent of substrate stereochemistry (Tables 11 and 16). In contrast, the V_{max} values were generally more than an order of magnitude lower for the NNAL substrates (Tables 12 and 16). Thus, NNK is clearly a much better substrate for CYP2A5 than NNAL (or the individual enantiomers) (Tables 11 and 16). Rat CYP2A3 metabolizes NNAL with an efficiency similar to mouse CYP2A5 and human CYP2A13, but the K_m values are slightly higher for the human enzyme (Table 16; Jalas *et al.*, 2003a).

In-vivo studies of NNK metabolism

The literature on in-vivo studies of NNK metabolism has been reviewed comprehensively (Hecht, 1998). Only selected studies are discussed below.

Monkey

Intravenous injection of NNK into patas monkeys resulted in rapid and extensive metabolism by α-hydroxylation and the formation of keto acid and hydroxy acid (Hecht *et al.*, 1993b). These metabolites accounted for a relatively large proportion of serum and urinary metabolites at all time-points. The other major metabolic pathway was reduction to NNAL, which was detected both unconjugated and as NNAL-*O*-Gluc, of which (*S*)-NNAL-*O*-Gluc predominated. NNAL-*O*-Gluc accounted for 15–20% of the urinary metabolites in monkeys given 0.1 μg/kg bw NNK, a dose similar to that of a smoker. Pharmacokinetic parameters for NNK metabolism were similar to those observed in baboons, which indicates rapid metabolism of NNK in primates (Adams *et al.*, 1985a). A comparison of pharmacokinetic parameters in rats, mice and hamsters showed that clearance of NNK varied predictably with body weight, similar to observations with NDMA, which implies that common phenomena govern the pharamacokinetics of these nitrosamines (Hecht *et al.*, 1993b).

Rat

Studies of the metabolism of NNK in rats consistently demonstrate rapid and extensive conversion of NNK to products of α-hydroxylation, pyridine-*N*-oxidation and carbonyl reduction. Less than 1% of the dose is excreted unchanged in the urine. Rapid metabolism of NNK is observed in all tissues examined; the highest amounts of α-hydroxylation are observed in the nasal mucosa, liver and lung, which are target tissues of NNK carcinogenesis (reviewed in Hecht, 1998).

The pharmacokinetics and metabolism of NNK (8.4 μmol/kg bw intravenously) were studied in bile duct-cannulated Fischer 344 rats (Wu *et al.*, 2002). After 24 h, approximately 85% of NNK was recovered (17.5% from bile and 67.6% from urine), which is consistent with previous studies (Schulze *et al.*, 1992; Hecht, 1998). Pharmacokinetic

Table 16. Steady-state kinetic parameters for human cytochrome P450 (CYP) 2A13-, rat CYP2A3- and mouse CYP2A5-mediated metabolism of NNAL

Species/ enzyme	Substrate	Metabolite	Kinetic parameters			Expression system	Reference
			V_{max}[a]	K_m (μM)	V_{max}/K_m[b]		
Human CYP2A13	(±)-NNAL	Lactol	1.50 ± 0.05	36 ± 3	0.042 ± 0.004	Baculovirus-infected Sf9 cells	Jalas *et al.* (2003a)
		Diol	0.79 ± 0.02	40 ± 3	0.020 ± 0.002		
		NNAL-*N*-oxide	0.12 ± 0.01	30 ± 7	0.0040 ± 0.0010		
Rat CYP2A3	(±)-NNAL	Lactol	0.41 ± 0.01	3.8 ± 0.5	0.11 ± 0.01		
		Diol	0.98 ± 0.02	16 ± 1	0.061 ± 0.004		
		NNAL-*N*-oxide	0.046 ± 0.003	2.6 ± 0.7	0.018 ± 0.005		
Mouse CYP2A5	(±)-NNAL	Lactol	0.55 ± 0.02	1.7 ± 0.3	0.32 ± 0.06	Baculovirus-infected Sf9 cells	Jalas & Hecht (2003)
		Diol	0.47 ± 0.01	5.1 ± 0.5	0.092 ± 0.009		
		NNAL-*N*-oxide	0.17 ± 0.01	4.7 ± 1.1	0.036 ± 0.009		
		NNK	0.78 ± 0.04	14 ± 1.8	0.056 ± 0.008		
	(*R*)-NNAL	Lactol	0.42 ± 0.02	2.1 ± 0.5	0.20 ± 0.05		
		Diol	1.2 ± 0.03	11 ± 0.8	0.11 ± 0.008		
		NNAL-*N*-oxide	ND				
		NNK	0.31 ± 0.03	16 ± 3.9	0.019 ± 0.005		
	(*S*)-NNAL	Lactol	0.45 ± 0.03	0.78 ± 0.26	0.58 ± 0.22		
		Diol	0.17 ± 0.01	1.3 ± 0.4	0.13 ± 0.04		
		NNAL-*N*-oxide	0.16 ± 0.01	1.1 ± 0.4	0.15 ± 0.05		
		NNK	0.78 ± 0.03	4.2 ± 0.6	0.19 ± 0.03		

K_m, Michaelis constant; ND, not detected; NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; NNK, 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone; Sf9, *Spodoptera frugiperda*; V_{max}, maximum velocity

[a] Units are pmol/min/pmol CYP.

[b] Units are pmol/min/pmol CYP/μM.

analysis indicated that NNK had a short urinary half-life (2.6 h), a large volume of distribution (321 ± 137 mL) and a total body clearance of 12.8 ± 2.0 mL/min. (*R*)-NNAL-Gluc was the major metabolite in bile and represented approximately 14% of the total NNK dose. Nearly all NNAL-Gluc were excreted as the (*R*)-diastereomer. The major metabolite in urine was keto acid (26% of the dose). Urinary excretion of NNAL and hydroxy acid comprised about 30% of the dose. Metabolism of NNK to (*S*)-NNAL appeared to favour α-hydroxylation of (*S*)-NNAL and stereoselective localization in the lung while metabolism of NNK to (*R*)-NNAL appeared to lead to detoxification through glucuronidation and biliary excretion (Wu *et al.*, 2002).

In a study designed to probe the effects of nicotine on NNK metabolism, Holzman rats received acute (36 h) or chronic (2 week) subcutaneous infusions of nicotine at rates that produced serum nicotine concentrations that were two to three times the venous nicotine concentrations usually measured in smokers (Keyler *et al.*, 2003). A single intraperitoneal dose of NNK (39 nmol/kg bw) was administered 24 h before the end of each infusion. Neither acute nor chronic nicotine infusion had any effect on the extent of NNK metabolism by α-hydroxylation; some small effects on pyridine-*N*-oxidation were observed. The results indicate that nicotine infusion has no effect on the carcinogenic pathways of NNK metabolism in the rat.

Mouse

A/J mice treated intraperitoneally with NNK (0.005–500 μmol/kg bw) excreted hydroxy acid (18–37% of urinary metabolites), keto acid (11–27%), (*R*)-NNAL-*O*-Gluc (ND–22%), NNAL-*N*-oxide (6–14%), NNK-*N*-oxide (ND–10%), NNAL (ND–29%) and 6-hydroxy-NNK (1%) in the 48-h urine, similar to results obtained in rats (Morse *et al.*, 1990b; Desai *et al.*, 1993; Hecht, 1998). At lower doses of NNK, levels of α-hydroxylation products increased while the levels of NNAL and NNAL-*O*-Gluc decreased. NNAL glucuronidation is quantitatively an unimportant metabolic pathway at low doses of NNK (Morse *et al.*, 1990b).

Following a 2-h nose-only exposure to mainstream cigarette smoke, mice were administered NNK intraperitoneally (7.5 μmol). Control mice were sham-exposed and treated with the same dose of NNK (Brown *et al.*, 2001a). The pattern of urinary metabolites was affected by smoke exposure. Mice exposed to smoke excreted 25% more NNAL, 15% less hydroxy acid and 42% less keto acid than control mice. Other metabolites (NNAL-Gluc and NNAL-*N*-oxide) were not affected. These results indicate that mainstream cigarette smoke causes a shift in metabolism which leads to lesser α-hydroxylation and greater excretion of NNAL.

Hamster

As in rats and mice, NNK is extensively metabolized by α-hydroxylation and other pathways in Syrian golden hamsters (Hecht, 1998). Female Syrian golden hamsters treated subcutaneously with NNK (80 nmol/kg) excreted 96% of the dose as urinary metabolites within 24 h of treatment (Richter & Tricker, 2002). α-Hydroxylation to keto acid (30.7% of

radioactivity in urine) and hydroxy acid (22.3%), and detoxification to NNAL-*N*-oxide (24.2%) accounted for almost 80% of the NNK metabolites in the 24-h urine. Smaller amounts of diol, HPB, NNAL-*O*-Gluc, NNK-*N*-oxide and NNAL were observed. Concurrent treatment with nicotine, cotinine or 2-phenethyl isothiocyanate (PEITC) decreased total α-hydroxylation from 58.1% (control) to 49.6% (nicotine), 41.2% (cotinine) and 54.6% (PEITC), with concomitant increases in NNAL (Richter & Tricker, 2002).

In-vivo studies of NNAL metabolism

Earlier studies demonstrate that the NNK/NNAL equilibrium favours NNAL in rodents and primates, and report some pharmacokinetic parameters for NNAL metabolism. Conversion of (*R*)-NNAL-*O*-Gluc to metabolites of NNAL and NNK was also observed (reviewed in Hecht, 1998).

Rat

Male Fischer 344 rats were treated intravenously with racemic [5-^{3}H] NNAL (8.5 μmol/kg) (Wu *et al.*, 2002). After 24 h, 43% of the dose was recovered from urine and 20% from bile. Urinary elimination half-lives were 9.5 h for NNAL, 7.8 h for hydroxy acid and 8 h for keto acid. Total body clearance of NNAL was 8.65 ± 2.6 mL/min and volume of distribution was 2772 ± 1423 mL. (*R*)-NNAL-Gluc was the major metabolite in bile and accounted for 16% of the NNAL dose. The urinary excretion profile following NNAL administration was similar to that of NNK except that less keto acid was excreted. Enantiomeric ratios for NNAL were evaluated in plasma, urine and bile. (*R*)-NNAL was the predominant form excreted. In the lung, 1 h after administration of racemic NNAL, 49% of the total metabolites was NNAL, with lesser amounts of NNAL-*N*-oxide (19%), hydroxy acid (10%) and keto acid (7%). NNAL levels in the liver were comparable with those in the lung; significant amounts of (*R*)-NNAL-Gluc and hydroxy acid were also observed. NNK was also identified in the lung and liver samples. Four hours after administration, NNAL remained the most abundant metabolite in the lung, but lower amounts were found in the liver and kidney. Enantiomeric ratios demonstrated that (*S*)-NNAL predominated in the lung and liver 4 h after administration. At 24 h after administration, the (*S*):(*R*) ratio was 57 in the lung, 1.2 in the liver and 3.4 in the kidney. NNAL made up 75% of total metabolites in the lung 24 h after administration. Collectively, the results demonstrated stereoselective excretion of (*R*)-NNAL-*O*-Gluc and retention of (*S*)-NNAL in the rat lung (Wu *et al.*, 2002).

Male Fischer 344 rats were treated intravenously with [5-^{3}H] (*R*)-NNAL (0.11–0.65 mg/kg) or (*S*)-NNAL (0.198–1.07 mg/kg) (Zimmerman *et al.*, 2004). After 24 h, 65% of the dose of (*S*)-NNAL was recovered (15% from the bile and 48% from urine), while almost 90% of the (*R*)-NNAL dose was recovered (44% from the bile and 45% from urine). Thus, excretion of (*S*)-NNAL was substantially less than that of (*R*)-NNAL, principally due to the difference in biliary excretion of (*R*)-NNAL-Gluc. The volume of distribution of (*S*)-NNAL (1792 ± 570 mL) was significantly greater than that of (*R*)-NNAL (645 ± 230 mL). Urinary elimination half-lives of metabolites were significantly shorter

after administration of (*R*)-NNAL (4.2 h) than after that of (*S*)-NNAL (6.3 h). Urinary metabolite profiles of the two enantiomers were markedly different. For (*R*)-NNAL, major metabolites consisted largely of unchanged NNAL, hydroxy acid and (*R*)-NNAL-Gluc. For (*S*)-NNAL, more keto acid and less hydroxy acid, more NNK-*N*-oxide and less NNAL were formed than in the case of (*R*)-NNAL; NNK was also observed. Tissue retention of metabolites demonstrated that a larger proportion of the dose was retained in the liver, lung and kidney 24 h after administration of (*S*)-NNAL compared with (*R*)-NNAL. There was significant and rapid conversion of (*S*)-NNAL to NNK in the tissues. At 24 h after administration of (*S*)-NNAL, 78% of lung metabolites was NNAL, with an (*S*):(*R*) ratio of 4.2, a shift from a ratio of 1.1 at 1 h after dosing, which indicated selective retention of (*S*)-NNAL in the lung. At 24 h after administration of (*R*)-NNAL, no quantifiable metabolites were found in the lung. These results demonstrate that (*R*)-NNAL, the less carcinogenic NNAL enantiomer (in mice), is excreted more rapidly than (*S*)-NNAL, while (*S*)-NNAL is selectively retained in the lung and is extensively converted to NNK.

Mouse

Racemic and enantiomeric NNAL as well as (*R*)-NNAL-Gluc (20 μmol) were administered intraperitoneally to female A/J mice and urine was collected (Upadhyaya *et al.*, 1999). The metabolites of racemic NNAL were hydroxy acid (10% of the dose), NNAL-*N*-oxide (3.1%), NNAL (20%) and (*S*)-NNAL-Gluc (16%). The profile of metabolites from the enantiomers was quite similar to that of racemic NNAL, except that (*S*)-NNAL was metabolized less effectively to (*R*)-NNAL-Gluc than (*R*)-NNAL. The formation of (*S*)-NNAL-Gluc from (*R*)-NNAL indicates that (*R*)-NNAL was converted to NNK *in vivo*. Minor amounts of urinary metabolites other than (*S*)-NNAL-Gluc were detected when (*S*)-NNAL-Gluc was administered to mice.

Haemoglobin adducts of NNK and NNAL

Adducts with globin are formed in rats by both the α-methylene and α-methyl hydroxylation pathways of NNK (Carmella & Hecht, 1987; Hecht, 1998). Depending on dose, species and protocol employed, approximately 15–40% of the pyridyloxobutyl adducts are released as HPB upon mild base hydrolysis (Carmella & Hecht, 1987; Murphy *et al.*, 1990a; Peterson *et al.*, 1990; Murphy & Coletta, 1993). The HPB-releasing adducts are esters, most probably with aspartate, glutamate or the terminal carboxy groups of globin (Carmella *et al.*, 1992). Pyridyloxobutyl cysteine adducts do not appear to be formed in measurable amounts in rats (Carmella *et al.*, 1990b). Co-incubation of rat hepatocytes and human red blood cells results in the formation of HPB-releasing adducts, as seen *in vivo* (Murphy & Coletta, 1993). Therefore, α-HOMeNNK or its glucuronide are sufficiently stable to migrate out of the hepatocyte and into the red blood cell. Human red blood cells alone can activate NNK but not to HPB-releasing adducts (Murphy & Coletta, 1993). In rats, both methyl and pyridyloxobutyl haemoglobin adducts increase linearly with the dose over a more than 3000-fold range (Murphy *et al.*, 1990a). DNA adducts in the liver and lung also increase over this range but the relationship with dose is non-linear.

The utility of HPB-releasing haemoglobin adducts as a biomarker of the overall carcinogenic effect of NNK was demonstrated in studies in which treatment of rats with PEITC or 6-phenylhexyl isothiocyanate resulted in a significant decrease in HPB-releasing adducts over an 18-month period compared with rats treated with NNK only; concurrently, a significant decrease in lung tumour induction was observed in the rats treated with isothiocyanates and NNK (Hecht *et al.*, 1996a,b). In rats treated with a mixture of benzo[*a*]pyrene (2 ppm in diet; 2 mg/kg) and NNK (2 ppm in drinking-water; 2 μg/mL), HPB-releasing haemoglobin adducts were significantly inhibited by concurrent treatment with dietary PEITC, or a mixture of PEITC and benzyl isothiocyanate, but not with benzyl isothiocyanate alone. Concurrently with the inhibition of HPB-releasing haemoglobin adducts, a decrease in HPB-releasing DNA adducts was observed in the rat lung, but not in the liver, as was an increase in the levels of NNAL and NNAL-Gluc excreted in urine (Boysen *et al.*, 2003). HPB-releasing haemoglobin adducts are also formed upon treatment of rats with NNAL (Hecht & Trushin, 1988).

DNA adducts of NNK and NNAL

In experimental systems, DNA adducts have been measured by high-performance liquid chromatography–radioflow detection after administration of [^{3}H]-labelled compounds, or by gas chromatography–mass spectrometry of released HPB. The latter technique has also been used in human studies (see Section 4.1.1(*a*)(iii)).

Studies of DNA adduct formation by NNK and NNAL have been extensively reviewed (Hecht, 1998). Highlights and recent studies are presented below.

In-vitro studies

All data indicate that there are two major types of NNK–DNA adducts: methyl adducts formed by α-methylene hydroxylation and pyridyloxobutyl adducts formed by α-methyl hydroxylation. Adduct formation is summarized in Figures 2 and 5. α-Methylene hydroxylation of NNK leads to the formation of Me-DZH and/or the methyldiazonium ion, which react with DNA to form 7-MedGuo, *O*^{6}-MedGuo and *O*^{4}-MedThd. Other adducts are probably also produced, based on studies of other methylating nitrosamines and nitrosoureas (Singer & Grunberger, 1983). DNA methylation by NNK is observed in a number of in-vitro studies with different systems that are capable of activating its metabolism, including rat lung cells and lung, liver or nasal mucosal microsomes (with added DNA), rat oral tissue and hamster lung (Devereux *et al.*, 1988; Rossignol *et al.*, 1989; Murphy *et al.*, 1990a; Guo *et al.*, 1991b; Hecht, 1998).

The chemistry of the intermediates that result from α-methyl hydroxylation of NNK has been studied in detail. Since α-HOMeNNK (Figure 2) is not very stable, this metabolite has been generated *in situ* by solvolysis of 4-(acetoxymethylnitrosamino)-1-(3-pyridyl)-1-butanone (NNKOAc) and 4-(carbethoxynitrosamino)-1-(3-pyridyl)-1-butanone. Extensive studies conclusively demonstrate that the major DNA adducts formed by this pathway *in vitro* and *in vivo*, which account for at least 50% of the DNA binding, release HPB (Figure 3) upon acid or neutral thermal hydrolysis (Hecht & Trushin, 1988;

Hecht *et al.*, 1988b; Spratt *et al.*, 1989; Murphy *et al.*, 1990a; Peterson *et al.*, 1990; Peterson & Hecht, 1991; Foiles *et al.*, 1992; Trushin *et al.*, 1994; Staretz *et al.*, 1997c; Hecht, 1998). These adducts are produced via POB-DZH but not by HPB itself (Hecht *et al.*, 1988). The HPB-releasing adducts have different stabilities in DNA, and are released in a triphasic manner (Peterson *et al.*, 1991b). Adducts that release HPB upon neutral thermal hydrolysis have recently been identified as 7(POB-1-yl)dGuo and O^2(POB-1-yl)dCyd (Wang *et al.*, 2003; Hecht *et al.*, 2004c). Other adducts formed by this pathway that have been identified are shown in Figures 2 and 5 (Haglund *et al.*, 2002; Wang *et al.*, 2003; Hecht *et al.*, 2004c). A similar group of adducts is formed upon methyl hydroxylation of NNAL (Figures 2 and 5; Upadhyaya *et al.*, 2003; Hecht *et al.*, 2004c).

Pyridyloxobutyl adducts inhibit O^6-alkylguanine–DNA alkyltransferase (AGT), the enzyme responsible for the repair of O^6-MedGuo. Since O^6-MedGuo is also formed by metabolic activation of NNK, this phenomenon is probably important in the persistence of O^6-MedGuo in NNK-exposed tissues (Peterson *et al.*, 1993).

In-vivo studies

Since O^6-methylguanine (O^6-MeGua) and 7-MeGua were first detected in the liver and lung of NNK-treated Fischer 344 rats (Castonguay *et al.*, 1984b), substantial research has been carried out on the occurrence and biological significance of methyl and pyridyloxobutyl adducts that result from the metabolic activation of NNK (Hecht, 1998).

Rat

Levels of 7-MeGua are 7.5–25 times higher than those of HPB released in the whole lung, depending on dose, with lower ratios at lower doses (Murphy *et al.*, 1990b). Levels of HPB-releasing adducts are about twice those of O^6-MeGua, which are about 10 times greater than those of O^4-MedThd (Belinsky *et al.*, 1986; Staretz *et al.*, 1997c). Consistently, the highest levels of O^6-MeGua and HPB-releasing adducts are found in Clara cells of the rat lung, with lower amounts in type II cells, macrophages and small cells (Belinsky *et al.*, 1987a; Belinsky *et al.*, 1988; Devereux *et al.*, 1988; Staretz *et al.*, 1997c). The dose–response relationship for adduct formation in whole lung and lung cell types is non-linear. Adduct levels at the lowest doses are higher than would be expected by linear extrapolation from higher doses, e.g. efficiency of alkylation increases considerably at low doses (Belinsky *et al.*, 1987a; Devereux *et al.*, 1988; Belinsky *et al.*, 1990; Murphy *et al.*, 1990b; Staretz *et al.*, 1997c). One interpretation of these data is the presence of CYPs in the rat lung, such as CYP2A3, which efficiently catalyse α-hydroxylation at low concentrations of NNK. A second interpretation relates specifically to O^6-MeGua. Pyridyloxobutyl adduct concentrations increase at low doses of NNK, which may lead to greater inhibition of AGT and consequent higher levels of O^6-MeGua (Peterson *et al.*, 1993).

During chronic treatment with high doses of NNK, O^6-MeGua increases consistently in the rat lung (Belinsky *et al.*, 1986). At lower doses over a 4-day period, O^6-MeGua persists to a greater extent in Clara cells than in other cell types (Belinsky *et al.*, 1988). This is partly because of lower levels of AGT in Clara cells than in other cell types after treatment with

NNK. Treatment with NNK inhibits AGT, which is probably due to DNA pyridyloxobutylation. However, when NNK was administered over the full 20-week course to induce lung tumours in rats, O^6-MeGua levels decreased by 82% in the Clara cells during the treatment period and were lower than those in the macrophages at 20 weeks (Staretz *et al.*, 1997c). The decrease is due to inhibition of CYP-catalysed α-methylene hydroxylation by NNK, as demonstrated in a study of α-methylene hydroxylation by isolated rat pulmonary microsomes after 20 weeks of treatment with NNK (Staretz *et al.*, 1997b).

Structure–activity studies suggest that both DNA methylation and pyridyloxobutylation are important in NNK-induced lung tumorigenesis in rats. Neither NDMA, which only methylates DNA, nor NNN, which pyridyloxobutylates but does not methylate DNA, is an effective lung carcinogen in rats (Hecht *et al.*, 1980a; Hoffmann *et al.*, 1984; Hecht *et al.*, 1986b). NNK yields greater amounts of O^6-MeGua than NDMA in Clara cells, but not in alveolar type II cells, and is metabolized somewhat more effectively to a pyridyloxobutylating agent than NNN in rat lung cells (Devereux *et al.*, 1988; Belinsky *et al.*, 1989a). Such differences in binding and metabolism may partially account for the distinctly greater pulmonary carcinogenicity of NNK than that of NDMA or NNN. However, only NNK provides the combination of DNA methylation and pyridyloxobutylation that appears to be critical for rat lung tumorigenesis. NNK-induced rat lung tumours arise in type II cells (Belinsky *et al.*, 1990). Levels of HPB-releasing adducts in type II cells of NNK-treated rats correlate with lung tumour incidence over a range of doses, which suggests that pyridyloxobutylation is an important pathway (Staretz *et al.*, 1997c). Levels of O^6-MeGua in Clara cells also correlate with lung tumour incidence over a wide dose range, which suggests some role of this adduct in spite of the fact that the Clara cell is probably not the cell of origin of the tumours (Belinsky *et al.*, 1990). The effects of PEITC on levels of HPB-releasing adducts in type II cells and other cell types of the lung correlate well with inhibition of NNK-induced lung tumorigenesis by PEITC, which provides further evidence for the importance of these adducts (Staretz *et al.*, 1997c). PEITC also inhibits O^6-MeGua levels in Clara cells to the same extent that it inhibits lung tumorigenesis, but this is not seen in other cell types (Staretz *et al.*, 1997c). PEITC selectively inhibits HPB-releasing DNA adducts in the lung, but not in the liver of NNK-treated rats (Boysen *et al.*, 2003). Collectively, the available data indicate that HPB-releasing adducts and O^6-MeGua are both important in lung tumour induction by NNK in rats.

Levels of DNA methylation in rat nasal mucosa are frequently higher than those in other tissues of NNK-treated animals (Belinsky *et al.*, 1986; Hecht *et al.*, 1986b; Belinsky *et al.*, 1987b). This is a consequence of the high NNK α-hydroxylation activity mediated by CYPs of the nasal mucosa. Although both α-methylene and α-methyl hydroxylation of NNK occur at similar rates in nasal mucosa microsomes (Smith *et al.*, 1992b), DNA methylation is greater than pyridyloxobutylation (Trushin *et al.*, 1994). These data indicate that, in rat nasal mucosa, α-HOMethyleneNNK is more effective as a DNA methylating agent than α-HOMeNNK is as a pyridyloxobutylating agent, possibly due to differences in the reactivity of the resulting alkylating agents, or to other factors such as glucuronidation of α-HOMeNNK (Hecht, 1998).

In spite of the relatively low levels of HPB-releasing adducts formed in rat nasal mucosa after treatment with NNK, these adducts appear to be important in tumour induction. NNK and NNN have similar carcinogenic activities toward the nasal mucosa, but NDMA has little activity (Hecht *et al.*, 1986b; Trushin *et al.*, 1994). NNK and NNN both pyridyloxobutylate nasal mucosa DNA to form HPB-releasing adducts (Trushin *et al.*, 1994). NNN does not methylate DNA whereas NDMA methylates but does not pyridyloxobutylate nasal DNA. These results support the role of DNA pyridyloxobutylation in rat nasal tumorigenesis. Studies with deuterated analogues of NNK further support this conclusion. Thus, [methylene-D_2]NNK is a stronger nasal carcinogen than either [methyl-D_3]NNK or NNK (Hecht *et al.*, 1987; Trushin *et al.*, 1994). Moreover, DNA pyridyloxobutylation by [methylene-D_2]NNK exceeds that by NNK while levels of *O*^{6}-MedGuo from [methylene-D_2]NNK are significantly lower than those from NNK or [methyl-D_3]NNK (Trushin *et al.*, 1994). Collectively, these data provide strong support for the proposal that DNA pyridyloxobutylation is critical in rat nasal carcinogenesis by NNK (Hecht, 1998).

Levels of 7-MeGua are 13–49 times greater than those of HPB-releasing adducts in rat liver, and levels of the latter are generally greater than those of *O*^{6}-MeGua (Murphy *et al.*, 1990b). The 7-MeGua:HPB-releasing adduct ratio at lower doses is lower in the liver than in the lung (Murphy *et al.*, 1990b). At low doses, levels of HPB-releasing adducts are lower in the liver than in the lung (Murphy *et al.*, 1990b; Boysen *et al.*, 2003). At high doses, the formation of 7-MeGua is saturated in the lung, but not in the liver, whereas the formation of HPB-releasing adducts is saturated in both tissues (Murphy *et al.*, 1990b). The higher levels of HPB-releasing adducts than of *O*^{6}-MeGua are probably due to differences in repair (Belinsky *et al.*, 1986, 1990; Peterson *et al.*, 1991b). During chronic NNK treatment, *O*^{6}-MeGua reaches a maximum in both hepatocytes and non-parenchymal cells, then declines rapidly due to induction of AGT (Belinsky *et al.*, 1986). The removal of HPB-releasing adducts from hepatic DNA appears to be slower than that of *O*^{6}-MeGua (Belinsky *et al.*, 1986; Peterson *et al.*, 1991b).

Treatment of rats with a single dose of NNK or NNAL resulted in the formation of 7-MeGua and *O*^{6}-MeGua (Hecht & Trushin, 1988). In hepatic DNA, levels of these adducts were similar 1–48 h after treatment with NNK or NNAL, while adduct levels in nasal mucosa and lung were somewhat higher after treatment with NNK than with NNAL. HPB-releasing adducts were formed with both NNK and NNAL in the liver; levels of the NNK-derived adducts were somewhat higher (Hecht & Trushin, 1998).

Mouse

Lung tumours are induced rapidly by a single dose of 10 μmol NNK in A/J mice (Hecht *et al.*, 1989). This model has been used extensively to examine mechanistic phenomena as well as the modifying effects of chemopreventive agents (Hecht, 1998). Levels of 7-MeGua are greater than those of *O*^{6}-MeGua, which in turn exceed those of HPB-releasing adducts (Peterson & Hecht, 1991). Levels of 7-MeGua and *O*^{6}-MeGua reached a maximum 4 h after injection of 10 μmol NNK, whereas the levels of released HPB were maximal after 24 h (Peterson & Hecht, 1991). Levels of 7-MeGua and released HPB

decrease with time, but O^6-MeGua is persistent, such that its levels exceed those of 7-MeGua 15 days after treatment (Peterson & Hecht, 1991). Levels of O^6-MedGuo were highest in type II cells and Clara cells, followed by small cells and whole lung (Devereux *et al.*, 1993).

Persistent O^6-MeGua is the critical determinant of lung tumour induction in A/J mice (Peterson & Hecht, 1991), but does not account for differences in sensitivity to NNK-induced lung tumorigenesis between A/J and C57BL/6 mice (Devereux *et al.*, 1993). In A/J mice, acetoxymethylmethylnitrosamine (AMMN), which can only methylate DNA, is highly tumorigenic whereas NNKOAc and NNN, which only pyridyloxobutylate DNA, are weakly active (Peterson & Hecht, 1991). [Methylene-D_2]NNK is significantly less tumorigenic than NNK or [methyl-D_3]NNK and also forms significantly less O^6-MeGua (Hecht *et al.*, 1990). Similarly, (4*R*)[4D]NNK is significantly less tumorigenic than either NNK or (4*S*)[4D]NNK, and also leads to lower levels of persistent O^6-MeGua (Jalas *et al.*, 2003b). There is an inflection in the dose–response curve for lung tumour induction by NNK in A/J mice, with an increase above a dose of 2–3 μmol NNK, at which persistent O^6-MeGua begins to be measurable (Peterson & Hecht, 1991). Evidently, AGT activity is saturated above this dose. Levels of O^6-MedGuo measured 96 h after treatment of A/J mice correlate strongly with tumour multiplicity, independent of the source of the methylating agent, e.g. NNK, AMMN or AMMN plus NNKOAc (Peterson & Hecht, 1991). In addition, GC→AT transitions in codon 12 of the K-*ras* gene are observed in a high percentage of lung tumours induced by NNK in A/J mice (see Section 4.4.2(*a*)(ii)), consistent with the importance of O^6-MeGua (Belinsky *et al.*, 1989b). Human methylguanine–DNA methyltransferase transgenic mice, which express high levels of AGT in the lung, were crossbred with A/J mice. Human AGT was expressed throughout the lung and, after treatment with NNK, these mice had lower levels of O^6-MeGua, lower tumour multiplicity and size of tumours in the lung and a lower frequency of K-*ras* mutations in the lung tumours than non-transgenic mice (Liu *et al.*, 1999).

The pyridyloxobutylation pathway is important in increasing the activity of the methylation pathway in A/J mouse lung tumorigenesis since NNKOAc markedly increases the tumorigenicity of AMMN over a wide dose range (Peterson & Hecht, 1991). NNKOAc enhances the persistence of O^6-MeGua in the lung of AMMN-treated mice due to the ability of HPB-releasing adducts to inhibit AGT (Peterson *et al.*, 1993; Liu *et al.*, 1996; Peterson *et al.*, 2001). The ability of NNKOAc to enhance the persistence of O^6-MeGua in the lung was similar to that of O^6-benzylguanine, a known inhibitor of AGT (Peterson *et al.*, 2001). The pyridyloxobutyl adduct, O^6(POB-1-yl)Gua, was detected in the liver, but not in the lung, of A/J mice treated with NNK (Thomson *et al.*, 2003). This adduct was also detected in the lung and liver of mice treated with NNKOAc, in the presence but not absence of O^6-benzylguanine, which indicates that O^6(POB-1-yl)Gua is repaired in part by AGT (Thomson *et al.*, 2003). Further studies demonstrated that O^6(POB-1-yl)Gua is repaired by mammalian AGT and that the rate of repair is highly dependent on protein structure (Mijal *et al.*, 2004). Inefficient repair of O^6(POB-1-yl)Gua by bacterial AGT explains the high mutagenic activity of this adduct in bacterial systems (Pauly *et al.*, 2002).

Adduct measurements have been made in A/J mouse liver after treatment with NNK, although tumour induction in this tissue is infrequent (Belinsky *et al.*, 1989b). As in the lung, the relative levels of adduct formation are 7-MeGua > O^6-MeGua > HPB-releasing adducts (Morse *et al.*, 1990a; Peterson *et al.*, 1990; Morse *et al.*, 1991). Levels of HPB-releasing adducts are higher in liver than in the lung, as are levels of 7-MeGua and O^6-MeGua (Morse *et al.*, 1990a; Peterson *et al.*, 1990; Morse *et al.*, 1991; Peterson & Hecht, 1991). The relatively high level of adducts in the liver is consistent with metabolic studies that show efficient α-hydroxylation with little or no pyridine-*N*-oxidation in this tissue, in contrast to the lung where pyridine-*N*-oxidation is a major competing detoxification pathway. Despite the high DNA adduct levels in A/J mouse liver, tumours are observed in the lung and this is clearly related to susceptibility factors inherent in this mouse strain (Hecht, 1998).

Hamster

Initial levels of 7-MeGua and O^6-MeGua are similar in rat and hamster liver after a single dose of NNK (Liu *et al.*, 1992). However, O^6-MeGua is repaired more rapidly in rats (half-time, 12 h), while only 14% of the initial O^6-MeGua is repaired 72 h after treatment in hamsters. 7-MeGua also persists longer in hamster than in rat liver. NNK rapidly depletes AGT in both rat and hamster liver, but AGT recovers in rats and not in hamsters. These results do not correlate with tumour induction by NNK. Whereas NNK is a weak hepatocarcinogen in rats, it does not induce liver tumours in hamsters. The results suggest that O^6-MeGua is not important in the hepatocarcinogenesis of NNK.

Other types of DNA damage

Single-strand breaks are observed in hepatocytes incubated with NNK and in the livers of NNK-treated animals (Hecht, 1998), and are probably produced by spontaneous or enzymatic depurination of adducts such as 7-MeGua or 7(POB-1-yl)Gua. Among the metabolites of NNK, keto aldehyde has received the most attention as a source of single-strand breaks. However, the single-strand breaks induced by keto aldehyde appear to have different properties (for example, pH dependence) from those caused by NNK, which indicates that NNK single-strand breaks do not result from keto aldehyde (Demkowicz-Dobrzanski & Castonguay, 1991). NNK single-strand breaks are repaired slowly and damage persists for 2–3 weeks after a single treatment with NNK in rats and hamsters (Jorquera *et al.*, 1994). One study demonstrated an increase in NNK-induced single-strand breaks in human lung cells after generation of superoxide by hypoxanthine/xanthine oxidase (Weitberg & Corvese, 1993).

Treatment with NNK causes increases in levels of the promutagenic adduct 8-oxodeoxyguanosine in mouse and rat lung, and in fetal liver following transplacental exposure of mice to NNK (Chung & Xu, 1992; Sipowicz *et al.*, 1997).

(iv) *Excretion*

Urine was the major route of excretion of NNK and NNAL metabolites with a pyridine ring in all studies with rodents and primates (Hecht, 1998).

In rats, 47% of a dose of [^{14}C-methyl]NNK was excreted in the expired air (Castonguay *et al.*, 1983b). From 7 to 17% of an NNK dose was excreted in the bile of rats, mainly as (*R*)-NNAL-*O*-Gluc (Schulze *et al.*, 1992; Wu *et al.*, 2002). After intravenous administration of individual NNAL enantiomers, metabolites of (*S*)-NNAL (15% of the total dose) and (*R*)-NNAL (44% of the total dose) were excreted in the bile of rats (Zimmerman *et al.*, 2004).

Male rhesus monkeys received a single intravenous dose of radioactive NNK (4.6–9.8 μg/kg) and urine was collected for 10 days (Meger *et al.*, 1999). Within the first 24 h, 86% of the dose was excreted. NNK-derived radioactivity was still detectable in urine 10 days after treatment. Patterns of metabolites in the urine during the first 6 h closely resembled those seen in patas monkeys (Hecht *et al.*, 1993b); end-products of NNK metabolic activation represented more than 50% of total radioactivity. At later time-points, the pattern shifted toward NNAL and NNAL-*O*-Gluc. There was no preferential biliary excretion of NNAL-Gluc compared with rats.

(*b*) *NNN*

(i) *Absorption*

Studies of the absorption, distribution, metabolism and excretion of NNN in experimental systems have been comprehensively reviewed (Hecht, 1998), and the reader is referred to that review for detailed coverage of the literature. Selected studies from this review which illustrate important points as well as more recent studies that are pertinent to the evaluation are presented below.

The penetration of NNN across porcine skin and various regions of the oral mucosa was determined. Specimens of porcine skin, keratinized gingival and non-keratinized mucosa from the floor of the mouth and cheek were studied. Skin showed a lower permeability than the oral regions, and the floor of the mouth was generally the most permeable site. The non-keratinized oral regions were most permeable to NNN (Squier, 1986). Concentrations of 25% ethanol and above significantly increased the permeability of oral mucosa to NNN, but this increase ceased with 50% ethanol. The permeability of oral mucosa to NNN was also increased by nicotine (0.2–2%). Combined use of nicotine and ethanol significantly increased the penetration of NNN across oral mucosa compared with ethanol alone (Du *et al.*, 2000). Permeability of rat skin and buccal mucosa to NNN was significantly increased in rats maintained on a diet that contained 6.7% ethanol compared with rats kept on an isocaloric diet without ethanol (Squier *et al.*, 2003).

(ii) *Distribution*

Whole-body and micro-autoradiographic studies of the distribution of NNN in rats, mice, marmoset monkeys and mini-pigs have been reviewed (Tjälve, 1991). In general, NNN is rapidly distributed throughout the body, which reflects an ability of this

compound to pass freely across biological membranes and distribute evenly in the intra- and extracellular tissue water. However, accumulation of radioactivity has been observed in certain tissues. In rats, 5 min after intravenous injection of [2′-^{14}C]NNN, the radioactivity was distributed homogeneously throughout most of the body and levels in tissues did not exceed the level in blood. However, high uptake was observed in a few tissues including the mucosa of the ethmoturbinates and the mucosa that covers the naso- and maxillo-turbinates. High radioactivity was also present in the submaxillary salivary glands, lacrimal glands, Zymbal glands, tarsal glands of the eyelids, preputial glands and stomach contents. The radioactivity in the nasal and tracheo-bronchial mucosa and the mucosa of the oesophagus and tongue was non-extractable, and a low level of non-extractable radioactivity was found in the liver, whereas that in other tissues was extractable (Brittebo & Tjälve, 1981). Whole-body autoradiography of mice treated intravenously with [2′-^{14}C]NNN showed a similar spectrum of tissue localization of bound metabolites as that in rats; the only difference was in the salivary glands which accumulated radioactivity in mice but not in rats (Brittebo & Tjälve, 1980; Waddell & Marlowe, 1980; Tjälve, 1991). Whole-body autoradiograms taken 15–220 min after intracardiac administration of [5-^{3}H]NNN to miniature pigs showed high levels of radioactivity in the mandibular and parotid salivary glands, Harder's gland, lacrimal glands, glands of the snout and respiratory part of the nasal cavity and melanin of the eyes and skin. Bound radioactivity was most abundant in the nasal mucosa and liver (Domellöf *et al.*, 1987). Autoradiograms obtained 4 h after intravenous injection of [2′-^{14}C]NNN into a marmoset monkey showed the highest levels of radioactivity in the liver, nasal mucosa, melanin of the eyes, hair follicles of the skin and ceruminous ear glands. Bound radioactivity was observed in the liver and nasal mucosa (Castonguay *et al.*, 1985a).

(iii) *Metabolism*

In-vitro studies

The in-vitro metabolism of NNN has been studied in the rat liver, oesophagus, nasal mucosa, oral tissue and lung, in hamster liver and oesophagus and in mouse lung; these studies have been reviewed (Hecht, 1998), and only selected studies and more recent investigations are discussed below.

Rat liver metabolizes NNN by hydroxylation at each position of the pyrrolidine ring, which results in the formation of HPB, lactol and other secondary metabolites, 3′-HONNN and 4′-HONNN. Myosmine and NNN-*N*-oxide have also been observed (Chen *et al.*, 1978; Hecht *et al.*, 1980b; Hecht, 1998).

In cultured rat oesophagus, which is a target tissue for NNN carcinogenesis, metabolites that result from the 2′-hydroxylation pathway of metabolic activation exceed those that result from the 5′-hydroxylation pathway of metabolic activation (Hecht *et al.*, 1982). DNA isolated from rat oesophagus cultured with [5-^{3}H]NNN contained HPB-releasing adducts (Murphy *et al.*, 1990a). Oesophageal microsomes metabolized NNN to HPB via 2′-hydroxylation and to lactol via 5′-hydroxylation. These reactions were probably mediated by a CYP enzyme, which had an apparent K_m of 49 μM (Murphy & Spina, 1994).

Products of 2′-hydroxylation exceeded those of 5′-hydroxlation by threefold, as in cultured rat oesophagus; however, in liver microsomes, the ratio of 2′:5′-hydroxylation varied between 0.23 and 0.71 depending on the concentration of NNN (Murphy & Spina, 1994). Cultured rat oesophagus metabolized (*S*)-NNN predominantly to products of 2′-hydroxylation while these products were significantly less prevalent in incubations with (*R*)-NNN. The 2′:5′-hydroxylation ratio ranged from 6.22 to 8.06 at various time intervals in the incubations with (*S*)-NNN, while the corresponding ratios were 1.22–1.33 in experiments with (*R*)-NNN (McIntee & Hecht, 2000). The CYP enzyme that is responsible for the metabolic activation of NNN in rat oesophagus has not been identified. One candidate was thought to be CYP2A3, which has been identified in small amounts in the rat oesophagus (Gopalakrishnan *et al.*, 2002). CYP2A3 is an efficient catalyst of NNN α-hydroxylation (K_m, 13 μM). However, metabolism of (*R*)- and (*S*)-NNN gives results that contrast to those observed in cultured rat oesophagus, which indicates that CYP2A3 is not involved in the catalysis of NNN α-hydroxylation in this tissue (Murphy *et al.*, 2000).

Cultured rat nasal mucosa, which is another target tissue of NNN carcinogenesis, metabolized NNN extensively by α-hydroxylation; pyridine *N*-oxidation was not observed (Brittebo *et al.*, 1983). HPB-releasing DNA adducts of NNN were detected in rat nasal mucosa cultured with NNN (Spratt *et al.*, 1989). Rat nasal mucosal microsomes catalysed both 2′- and 5′-hydroxylation of NNN, with low K_m values of 2–3 μM. NNN inhibited coumarin 7-hydroxylation, which suggests the involvement of a CYP2A enzyme (Patten *et al.*, 1998).

Cultured rat oral tissue metabolized NNN in a fashion similar to that observed with cultured rat oesophagus, but HPB-releasing DNA adducts were not observed (Murphy *et al.*, 1990a). NNN metabolism was inhibited by nicotine and, to a lesser extent, by NNK (Murphy & Heiblum, 1990).

There is some consistency between the ratios of 2′:5′-hydroxylation in different rodent tissues and their susceptibility to carcinogenesis by NNN (Hecht, 1998). The 2′:5′-hydroxylation ratio is typically 2–4 in rat oesophagus and nasal mucosa, which are the main target tissues of NNN in the rat. In the liver, which is a non-target tissue, 2′:5′-hydroxylation ratio is 0.3–1.4. Hamster oesophagus, which is a non-target tissue, predominantly 5′-hydroxylates NNN (Hecht *et al.*, 1982). These results are consistent with a role for 2′-hydroxylation in tumour induction by NNN and for 5′-hydroxylation in detoxification. In contrast, 5′-hydroxylation and 2′-hydroxylation occur to equal extents in hamster trachea (McCoy *et al.*, 1982) and 5′-hydroxylation exceeds 2′-hydroxylation in A/J mouse lung (Castonguay *et al.*, 1983a), both of which are target tissues of NNN carcinogenesis (Hecht, 1998).

Rat CYP2A3 and mouse CYP2A5 catalyse 5′-hydroxylation of both enantiomers of NNN with low K_m values (0.74–3.35 μM). Mouse CYP2A4 is a poorer catalyst with K_m values of 54.1–68.5 μM. Rat CYP2A3 and mouse CYP2A5 catalyse 2′-hydroxylation of (*R*)-NNN with K_m values of 0.73–1.64 μM, while the K_m for mouse CYP2A4 is 66 μM. 2′-Hydroxylation of (*S*)-NNN was not observed in studies with these enzymes (Wong *et al.*, 2005b).

2′- and 5′-Hydroxylation of NNN can lead to DNA damage. 2′-Hydroxylation generates the same intermediate — POB-DZH (Figure 6) — as methyl hydroxylation of NNK (Hecht, 1998; Wang *et al.*, 2003; Hecht *et al.*, 2004c). Formation of HPB-releasing DNA adducts is therefore expected following 2′-hydroxylation of NNN and this been observed in cultured rat oesophagus and nasal mucosa. Adducts could also be formed from 5′-HONNN via *iso*-POB-DZH, but this has not been reported (Hecht, 1998).

In-vivo studies

The effect of NNN on hepatic and pulmonary carcinogen metabolizing enzymes in male Sprague-Dawley rats was evaluated in a series of studies that used the following basic experimental protocol. Inbred male weanling Sprague-Dawley rats (19–21 days of age and weighing 35–50 g) were randomly divided into three groups of eight animals each and were placed on three different dietary regimens that consisted of a standard diet, a control semisynthetic diet and a semisynthetic deficient diet. In each set of experiments, the semisynthetic diets were either adequate (control) or deficient in vitamin A (Nair *et al.*, 1991), vitamin B complex (Ammigan *et al.*, 1990a) or protein (Ammigan *et al.*, 1989). At 12 weeks, 75% of the dose that causes 50% lethality (LD_{50}) was divided into three equal doses and was given intraperitoneally at 24-h intervals. Twenty-four hours after the last injection, overnight fasted animals were killed and the lung and liver were excised. Hepatic and pulmonary biotransformation enzymes, CYPs, cytochrome b-5, benzo[*a*]-pyrene hydroxylase, benzphetamine *N*-demethylase, glutathione *S*-transferase (GST) and glutathione (GSH) content were determined. Vitamin A and C were also determined.

NNN was more toxic to animals with nutritional deficiencies. In the vitamin A-, B complex- or protein-deficient rats, the LD_{50} of NNN was reduced by 20–24% (Ammigan *et al.*, 1990b). These deficiencies resulted in a decrease in the basal levels of CYPs, benzo[*a*]-pyrene hydroxylase, benzphetamine demethylase, GST and GSH.

In vitamin A-sufficient and -deficient groups, treatment with NNN significantly increased the levels of phase I-activating enzymes in all treatment groups. A higher increase in hepatic and pulmonary phase I activities was observed in the deficient animals compared with the sufficient groups. An increase in the GSH/GST system was observed in the sufficient group following treatment; however, in the deficient animals, exposure to the NNN caused suppression of the hepatic and pulmonary GSH/GST systems (Nair *et al.*, 1991).

When groups of Sprague-Dawley rats fed low-protein (5% casein) or vitamin B complex-deficient diets were exposed to NNN by the same protocol, a significant increase in phase I enzymes with concurrent inhibition of the GSH/GST levels was observed compared with the corresponding control groups fed high-protein (20% casein) or vitamin B complex-sufficient diets (Ammigan *et al.*, 1989, 1990a). The hepatic pool of vitamin A was depleted while that of vitamin C was increased in Sprague-Dawley rats fed low-protein diet and exposed to NNN. Altered metabolism resulting from vitamin deficiency and/or protein–calorie malnutrition may be an important factor in the modulation of the metabolism of NNN.

The metabolism of NNN has been studied in rats, hamsters, mice, monkeys and mini-pigs (reviewed in Hecht, 1998). NNN is rapidly metabolized and eliminated primarily in urine. Hydroxy acid via 5′-hydroxylation and keto acid via 2′-hydroxylation are the major urinary metabolites of NNN in rodents, marmoset monkeys and mini-pigs (Hecht, 1998). Other metabolites that are consistently observed in the urine are NNN-*N*-oxide and norcotinine (Hecht, 1998). In patas monkeys, the major urinary metabolites are hydroxy acid, 3′-Honorcotinine, 3′-HOnorcotinine-Gluc, norcotinine-*N*-oxide and norcotinine (Upadhyaya *et al.*, 2002). Small amounts of unchanged NNN are also observed in the urine of treated animals (Hecht, 1998).

In rats treated with racemic NNN, (*S*)-hydroxy acid and (*R*)-hydroxy acid represented 36% and 64% of total hydroxy acid in the urine, respectively (Trushin & Hecht, 1999). Products of 2′-hydroxylation predominated in the urine of rats treated with (*S*)-NNN while products of 5′-hydroxylation were more prevalent in the rats treated with (*R*)-NNN (McIntee & Hecht, 2000).

Haemoglobin adducts

HPB-releasing haemoglobin adducts are formed in rats treated with NNN. The adduct levels are only about 16% of those induced by NNK (Carmella & Hecht, 1987).

DNA adducts

HPB-releasing adducts are present in acid or enzyme hydrolysates of hepatic DNA from NNN-treated rats, in acid hydrolysates of pulmonary DNA from NNN-treated mice and in acid hydrolysates of DNA from the respiratory and olfactory parts of the nasal mucosa of rats treated with NNN; the levels in the respiratory mucosa are higher (reviewed in Hecht, 1998). As may be expected, O^6-MeGua is not detected in the nasal mucosa or liver of rats treated with NNN (Castonguay *et al.*, 1985b).

(iv) *Excretion*

Urine is the major route of excretion of NNN and metabolites in rodents and accounts for 60–80% of the dose (Hecht, 1998).

Urine samples were collected from seven groups of eight Sprague-Dawley rats that were maintained on semisynthetic diets sufficient or deficient in vitamin A, sufficient or deficient in vitamin B complex and sufficient or deficient in protein, or on standard control diet. All groups were exposed to NNN. Urine was tested for mutagenic activity using the *Salmonella*/microsome assay. A higher mutagenic activity of urine was observed in the exposed groups on each of the deficient diets. The order of mutagenicity of all treatments was deficient diet > standard diet > nutritionally sufficient diet (Ammigan *et al.*, 1990b). Thus, NNN-exposed animals probably have greater exposure to mutagenic metabolites, which are generated by increased phase I enzymes and decreased detoxification system.

(*c*) *NAB*

(i) *Absorption*

No data were available to the Working Group

(ii) *Distribution*

No data were available to the Working Group

(iii) *Metabolism*

In rats treated by gavage with [2′-^{14}C]NAB, 68.5% of the dose was excreted in the urine (Hecht & Young, 1982). 5-Hydroxy-5-(3-pyridyl)pentanoic acid, formed by 6′-hydroxylation, and NAB-*N*-oxide were detected as urinary metabolites. In cultured rat oesophagus treated with [2′-^{14}C]NAB , 5-hydroxy-5-(3-pyridyl)pentanoic acid, formed by 6′-hydroxylation, was a major metabolite and 5-oxo-5-(3-pyridyl)pentanoic acid, formed by 2′-hydroxylation, was a minor metabolite at all time-points examined. These results contrasted with those obtained by rat oesophageal metabolism of NNN, in which 2′-hydroxylation predominated (Hecht & Young, 1982).

(iv) *Excretion*

Urine was the major route of excretion of NAB metabolites in the rat (Hecht & Young, 1982).

(*d*) *NAT*

(i) *Absorption*

No data were available to the Working Group.

(ii) *Distribution*

No data were available to the Working Group.

(iii) *Metabolism*

In Fischer 344 rats, the pharmacokinetics of NAT fit a two compartment model. Pharmacokinetic parameters were: half-life, 540 min; blood clearance, 128 mL/h; and apparent volume of distribution, 695 mL (Adams *et al.*, 1985b).

(iv) *Excretion*

No data were available to the Working Group.

4.2 Toxic effects

4.2.1 *Humans*

No data were available to the Working Group

4.2.2 *Experimental systems*

(*a*) *NNK and NNAL*

(i) *Animals*

NNK induced cytotoxicity in the nasal passage of male Fischer 344 rats: it damaged Steno's and Bowmans's glands and caused degeneration of the olfactory epithelium at intraperitoneal doses of 4.8–48 μmol/kg bw daily for up to 12 days (Belinsky *et al.*, 1987b). NNK also induced mild centrilobular necrosis in the liver, which progressed to collapse of the centrilobular architecture at higher doses (Belinsky *et al*., 1986). An intraperitoneal dose of 0.39 mmol/kg bw induced an increase in levels of alanine transaminase, aspartate transaminase and lactate dehydrogenase in male Syrian golden hamsters over 2–3 weeks (Jorquera *et al.*, 1994).

(ii) *In-vitro cellular systems*

NNK induced cytotoxicity in rat tracheal epithelial (RTE) cells at concentrations of 100–200 μg/mL (Zhu *et al*, 1991) and in human–hamster hybrid A_L cells at a dose of 500 μg/mL (Zhou *et al.*, 1999). NNK caused dose- and time-dependent toxicity in hamster pancreatic duct cells *in vitro* (Baskaran *et al.*, 1994).

No data on NNAL were available to the Working Group.

(*b*) *NNN*

The subcutaneous LD_{50} of NNN in male rats observed for 8 days was > 1000 mg/kg bw. In rats that died, haemorrhages were observed in the lungs and abdominal organs and epithelial-cell necrosis in the posterior nasal cavities and liver (Hoffmann *et al*., 1975). The LD_{50} determined in 12-week-old Sprague-Dawley rats was 200 mg/kg bw with a standard diet and 190 mg/kg bw for animals fed vitamine B- and protein-deficient diets (Ammigan *et al.*, 1990b).

(*c*) *NAB*

The subcutaneous LD_{50} of NAB in Fischer rats was > 1000 mg/kg bw (Hoffmann *et al*., 1975).

(*d*) *NAT*

No data were available to the Working Group.

4.3 Reproductive and developmental effects

No data were available to the Working Group.

4.4 Genetic and related effects

4.4.1 *Humans*

DNA adducts as biomarkers of exposure to NNK and NNN are discussed in Section 4.1. No data were available for NNAL, NAB or NAT.

4.4.2 *Experimental systems*

(*a*) *NNK and NNAL*

(i) *NNK*

DNA adducts as biomarkers of exposure to NNK have been discussed in Section 4.1.2.(*a*)(iii).

Mutagenicity, clastogenicity, cell transformation and other effects

Mutagenicity and allied effects (for details and references, see Table 17)

In-vitro studies

NNK caused a dose-dependent increase in mutations in *S. typhimurium* strains TA100 and TA1535 in the presence of a liver microsomal preparation from Aroclor-1254-induced rats.

Primary hepatocytes, a liver metabolic activation system (S9 fraction), and tracheal epithelial cells from normal and Aroclor-1254-induced rats were compared for bio-activation of NNK in the *Salmonella* mutagenicity assay. Without activation, NNK was not mutagenic in *S. typhimurium* TA1535. The bioactivation of NNK to a mutagenic metabolite was achieved by incubation with the liver S9 metabolic activation system from Aroclor-1254-induced rats or with primary hepatocytes from both untreated and Aroclor-1254-pretreated rats. In contrast, NNK incubated with rat tracheal epithelial cells from both uninduced or Aroclor-1254-induced rats produced no measurable mutagenic activity in strain TA1535 (Zhu *et al.*, 1991).

NNK was mutagenic in a *Salmonella* tester strain that carries the human *CYP2A6* and human NADPH-*CYP reductase* (YG7108 *2A6*/*OR*) in the absence of an exogenous metabolic activation system. In another report, NNK was shown to be mutagenic in strain TA7004 with rat and hamster metabolic activation systems, but not in TA100. It was also mutagenic in *S. typhimurium* TA98.

NNK has been used as a model mutagen in several studies that employed the *S. typhimurium* mutagenicity assay to determine the anti-mutagenic properties of various compounds (reviewed in Hecht, 1998).

NNK was mutagenic in the Mutatox test using the dark mutant M-169 of *Vibrio fischeri* (Yim & Hee, 2001).

In primary rat hepatocytes and rabbit lung cells, NNK induced DNA strand breaks without exogenous activation and unscheduled DNA synthesis.

Table 17. Genetic and related effects of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)

Test system	Result[a]		Dose[b] (LED or HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Salmonella typhimurium TA100, TA1535, reverse mutation	NT	+	2 µmol/plate [414 µg/plate]	Hecht *et al.* (1983c)
Salmonella typhimurium TA100, TA1535, reverse mutation	NT	+	2 µmol/plate[c] [418 µg/plate]	Hecht *et al.* (1983c)
Salmonella typhimurium TA100, TA1535, reverse mutation	NT	–	4 µmol/plate[d] [840 µg/plate]	Hecht *et al.* (1983c)
Salmonella typhimurium TA100, reverse mutation	–	+	1000 µg/plate	Padma *et al.* (1989b)
Salmonella typhimurium TA100, reverse mutation	–	–	2000 µg/plate	Yim & Hee (2001)
Salmonella typhimurium TA1535, reverse mutation	–	+	1000 µg/mL	Padma *et al.* (1989b)
Salmonella typhimurium TA1535, reverse mutation	–		2500 µg/plate	Zhu *et al.* (1991)
Salmonella typhimurium TA1535, reverse mutation		+	500 µg/plate	Zhu *et al.* (1991)
Salmonella typhimurium TA1538, reverse mutation	–	–	1000 µg/mL	Padma *et al.* (1989b)
Salmonella typhimurium TA98, reverse mutation	–	–	1000 µg/plate	Padma *et al.* (1989b)
Salmonella typhimurium TA98, reverse mutation	-	+	20 µM [4.2 µg/mL]	Kolar & Lawson (1997)
Salmonella typhimurium YG7108, reverse mutation	–	NT	0.7 mM [145 µg/mL]	Kushida *et al*. (2000a,b)
Salmonella typhimurium YG7108 -2A6/OR[e], reverse mutation	+	NT	0.1 mM [20.7 µg/mL]	Kushida *et al*. (2000a,b)
Salmonella typhimurium YG7108 -2E1/OR[f], reverse mutation	–	NT	0.7 mM [145 µg/mL]	Kushida *et al*. (2000a,b)
Salmonella typhimurium TA7004, reverse mutation	–	+	2000 µg/plate	Yim & Hee (2001)
DNA strand breaks, primary rat hepatocytes *in vitro*	+	NT	5 mM [1035 µg/mL]	Liu *et al.* (1990)
DNA strand breaks, primary rat hepatocytes *in vitro*	+	NT	6.25 µmol/mL [1297 µg/mL]	Pool-Zobel *et al.* (1992)
DNA strand breaks, isolated rabbit lung cells (Type II and Clara) *in vitro*	+	NT	30 µM [6.22 µg/mL]	Becher *et al.* (1993)
DNA strand breaks, isolated rabbit alveolar macrophages *in vitro*	–	NT	300 µM [62.2 µg/mL]	Becher *et al.* (1993)
Unscheduled DNA synthesis, freshly isolated rat hepatocytes *in vitro*	+	NT	1 mM [207.5 µg/mL]	Williams & Laspia, 1979
Unscheduled DNA synthesis, rabbit lung cells *in vitro*	+	NT	2 mM [414.5 µg/mL]	Dahl *et al.* (1990)

Table 17 (contd)

Test system	Result[a]		Dose[b] (LED or HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Gene mutation, Chinese hamster lung fibroblastic V79 cells, *Hprt* locus, *in vitro*	–	+	10 mM [2072 µg/mL]	Swedmark *et al.* (1994)
Sister chromatid exchange, Chinese hamster lung fibroblastic V79 cells *in vitro*	–	+	20 µg/mL	Zimonjic *et al.* (1989)
Sister chromatid exchange, Chinese hamster lung fibroblastic V79 cells *in vitro*	–	+	20 mM [4145 µg/mL]	Alaoui Jamali *et al.* (1988)
Sister chromatid exchange, Chinese hamster ovary (CHO) cells *in vitro*	NT	+	0.1 mM [20.7 µg/mL]	Lee *et al.* (1996)
Micronucleus formation, Chinese hamster lung fibroblastic V79 cells *in vitro*	–	+	5 mM [1036 µg/mL]	Alaoui Jamali *et al.* (1988)
Micronucleus formation, rat tracheal epithelial cells *in vitro*	+	NT	50 µg/mL	Zhu *et al.* (1991)
Chromosomal aberrations, Chinese hamster lung fibroblastic V79 cells *in vitro*	+	–	20 mM [4145 µg/mL]	Alaoui Jamali *et al.* (1988)
DNA strand breaks, human MRC-5 fetal lung cells *in vitro*	+	NT	5 mM [1035 µg/mL]	Weitberg & Corvese (1993, 1997)
Gene mutation, human lymphoblastoid cells, *HPRT* locus, *in vitro*	+	NT	1 µg/mL	Krause *et al.* (1999)
Sister chromatid exchange, human peripheral blood lymphocytes *in vitro*	+	NT	[0.48 mM] 100 µg/mL	Padma *et al.* (1989b)
Sister chromatid exchange, human peripheral blood lymphocytes *in vitro*	+	+	[0.096 mM] 20 µg/mL	Zimonjic *et al.* (1989)
Micronucleus formation, human AGT repair-deficient fibroblasts *in vitro*	+	NT	0.05 mM [10.4 µg/mL]	Pohlmann *et al.* (1992)
Micronucleus formation, human AGT repair-proficient fibroblasts *in vitro*	–	NT	1 mM [207.5 µg/mL]	Pohlmann *et al.* (1992)
Micronucleus formation, human-derived hepatoma HepG2 and Hep3B cells *in vitro*	–	NT	5 mM [1036 µg/mL]	Majer *et al.* (2004)
Chromosomal aberrations, human peripheral blood lymphocytes *in vitro*	+	NT	0.48 mM [100 µg/mL]	Padma *et al.* (1989b)

Table 17 (contd)

Test system	Result[a]		Dose[b] (LED or HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
DNA strand breaks, rat hepatocytes *in vivo*	+		12.5 mg/kg bw po 1× [60 µmol/kg bw]	Pool-Zobel *et al.* (1992)
DNA strand breaks, rat and hamster hepatocytes *in vivo*	+		0.39 mmol/kg bw [81 mg/kg bw] ip 1×	Jorquera *et al.* (1994)
Gene mutation, rat splenic T lymphocytes, *Hprt* locus, *in vivo*	–		150 mg/kg bw ip 1×	Jansen *et al.* (1996)
Micronucleus formation, Swiss male mice bone marrow *in vivo*	+		250 mg/kg bw/d ip 2×	Padma *et al.* (1989b)
Micronucleus formation, rat tracheal epithelial cells *in vivo*	+		150 mg/kg bw/d ip 3×	Zhu *et al.* (1991)

[a] +, positive;–, negative; NT, not tested
[b] LED, lowest effective dose; HID, highest ineffective dose; d, day; ip, intraperitoneal injection
[c] [4,4-dideutero]NNK
[d] [4-trideutero]NNK
[e] Co-expressing human CYP 2A6 together with human NADPH-CYP reductase (OR)
[f] Co-expressing human CYP 2E1 together with human NADPH-CYP reductase (OR)

NNK was mutagenic in V79 hamster cells when assayed with a metabolic activation system obtained from ethanol-treated pancreatic duct epithelial cells from humans and Syrian hamsters and from ethanol-treated CK cells (immortalized hamster pancreatic duct epithelial cells) (Kolar & Lawson, 1997). It induced mutations at the *Hprt* locus in V79 Chinese hamster cells co-cultivated with rat hepatocytes that provided metabolic activation.

NNK caused sister chromatid exchange in Chinese hamster ovary cells in the presence of metabolic activation in three studies.

NNK induced micronucleus formation in Chinese hamster lung fibroblastic V79 cells in the presence of a metabolic activation system. Micronuclei were also induced in V79 cells by NNK activated by fetal liver and lung homogenates from rat fetuses on the 15th day of the gestation (Alaoui Jamali *et al.*, 1998). In rat tracheal epithelial cells treated with NNK, micronuclei were formed without a metabolic activation system.

NNK induced chromosomal aberrations without exogenous bio-activation in Chinese hamster lung fibroblastic V79 cells.

NNK induced a dose-dependent toxicity in human–hamster hybrid A_L cell assay. Treatment with NNK at a low dose, when combined with radon α-particles, resulted in a combined mutagenic effect that was consistent with an additive model, but less than additive at higher concentrations of NNK (Zhou *et al.*, 1999). In mammalian cells, NNK induced mostly deletions (Zhou *et al*., 1999).

NNK induced DNA strand breaks in fetal human lung cells. NNK induced mutations at the *HPRT* locus in human lymphoblastoid MCL-5 cells without metabolic activation. In human lymphocytes, NNK induced sister chromatid exchange without exogenous bioactivation in two studies. Micronucleus formation was observed in AGT repair-deficient, but not in repair-proficient human fibroblasts in the absence of exogenous activation. NNK did not induce micronuclei in human hepatoma cell lines HepG2 and Hep3B. NNK induced chromosomal aberrations without exogenous bioactivation in human lymphocytes.

In-vivo studies

NNK did not induce mutations in rat splenic T-lymphocytes *in vivo*. NNK induced DNA strand breaks in the hepatocytes of rats and hamsters (see Section 4.1.2(*a*)(iii)), and micronucleus formation in the bone marrow of male Swiss mice.

Mutations in transgenic systems

In vitro, human CYP2A6 was lipofected via a retroviral vector in AS52 Chinese hamster ovary cells, which contain the bacterial *gpt* gene that can be mutated to 6-thioguanine resistance. At the highest dose of NNK (1200 μg/mL), a 14-fold (339×10^{-6}) increase in mutant frequency was observed in AS52-E8 cells compared with the spontaneous frequency of 24×10^{-6} (Tiano *et al*., 1994).

In vivo, NNK was administered intraperitoneally to *lacZ* transgenic mice (Muta^{TM}Mouse) at 125 and 250 mg/kg bw once a week for 4 weeks. The mutant frequencies in the *lacZ* and *cII* genes from lung and liver increased dose-dependently up to 10-fold compared with the controls. The proportion of G:C→A:T transition mutations in

the total number of mutants was less than the number of A:T→T:A and A:T→C:G transversions (Hashimoto *et al*., 2004). NNK was also mutagenic in a mixture of pooled oral tissues (gingival, buccal, pharyngeal and sublingual), and in tongue and lung tissue of *lacZ* transgenic mice (Muta™ Mouse) (von Pressentin *et al*., 1999).

K-*ras* and *TP53* mutations

DNA isolated from 20 lung hyperplasias obtained after treatment of adult A/J mice with NNK was screened for the presence of activated K-*ras*. This gene was activated in 17/20 lesions; 85% of the mutations were a G:C→A:T transition within codon 12 (GGT→GAT), a mutation that is consistent with base mispairing produced by the formation of the O^6-MeGua adduct (Belinsky *et al*., 1992).

Activated K-*ras* gene was detected in 100% of lung tumours induced in C3H mice by treatment with NNK (50 mg/kg bw); the activating mutation detected in all samples was a G:C→A:T transition (GGT→GAT) in codon 12 (Devereux *et al*., 1991). NNK caused GGT→GAT mutations in codon 12 of K-*ras* gene in lung tumours induced in A/J mice (Chen *et al*., 1993; Ronai *et al*., 1993).

The relationship between the development of peripheral lung lesions induced by NNK and K-*ras* gene mutation, and the correlations between histological alterations and the course of lung lesion development after treatment with NNK and K-*ras* gene mutation were investigated in A/J mice. K-*ras* gene mutations were identified in seven of 12 (58.3%) hyperplasias, in 42/56 (75.0%) adenomas and in three of four (75.0%) adenocarcinomas. The most frequent K-*ras* gene mutation was a G→A transition at the second base of codon 12, which accounted for 86.5% of all the mutations detected (Kawano *et al*., 1996).

Analysis of lung tumour DNA from A/J mice treated with NNK indicated that 15/17 (88%) samples contained G→A transitions at the second base of codon 12 in the K-*ras* gene. Similarly, in lung tumours from (A/J × TSG-*p53*)F_1 hybrid mice treated with NNK, 29/30 (97%) contained G→A transitions at the second base of codon 12 of the K-*ras* gene. No mutations of the *p53* gene were found in any of the tumours analysed, which suggests minimal involvement of this gene in the development of lung adenomas. The *p53* allele in (A/J × TSG-*p53*)F_1 mice does not alter the incidence or multiplicity of NNK-induced lung tumours (Matzinger *et al*., 1995).

Lung tumours induced by subcutaneous injection of NNK into Syrian golden hamsters were examined for mutations in the K-*ras* oncogene and the *TP53* tumour-suppressor gene by direct sequencing. The K-*ras* mutation frequency in RNA isolated from pooled tumours and that in DNA isolated from individual tumours were found to be identical. Activated K-*ras* alleles were detected in 77–94% of tumours. All mutations observed except one (from a total of 65), at either codon 12 or 13, were G:C→A:T. No mutations were detected at codon 61. Examination of the same tumours for *TP53* mutations showed only one point mutation. Treatment of Syrian golden hamsters with NNK resulted in a distinct mutation pattern in the K-*ras* gene whereas *TP53* gene mutations may not play a major role at this stage in hamster lung tumorigenesis (Oreffo *et al*., 1993).

Gene expression profile

Characteristic expression profiles induced by NNK at a dose of 20 mg/kg bw per day were investigated in rat liver for 14 days. Fourteen genes that are involved in DNA-damage response (five), detoxification response (six) and cell survival/proliferation (three) were up-regulated and one gene each that is involved in mitochondrial damage and dedifferentiation were down-regulated (more than two-fold). O^6-MeGua–DNA methyltransferase was among one of the genes that were up-regulated. Increased expression profiles were weakly detectable at day 1 and then increased with time (Ellinger-Ziegelbauer, 2004).

Cytotoxicity and cell transformation

The cytotoxicity and transforming activity of NNK was studied by the assays of colony-forming efficiency, micronucleus formation and cell transformation in rat tracheal epithelial cells both *in vitro* and *in vivo*. Results from the in-vitro experiments indicated that low concentrations of NNK (0.01–25 μg/mL) caused increases in colony-forming efficiency of rat tracheal epithelial cells from 15% to more than 100%. At higher concentrations (100–200 μg/mL), NNK was significantly toxic to these cells. Treatment with NNK *in vitro* (50–200 μg/mL) significantly increased the transformation frequency in four of five (50 μg/mL) and six of eight (100 μg/mL) experiments. The in-vivo exposure of rats to NNK (150–450 mg/kg intraperitoneally) resulted in a 60–85% reduction in colony-forming efficiency in rat tracheal epithelial cells (Zhu *et al*., 1991).

Immortalized human bronchial epithelial cells (BEAS-2B cells) grown in de-epithelialed rat tracheas were exposed to NNK and subcutaneously transplanted into athymic nude mice. The cells were neoplastically transformed to produce invasive adenocarcinoma with phenotypic changes similar to the progressive changes that occur during human lung carcinogenesis (Klein-Szanto *et al.*, 1992).

In-vitro transformation of spontaneously immortal hamster pancreatic duct cells has been described following exposure to 20 mM NNK for 1, 3, 5 and 7 days. Cells treated with NNK grew as a monolayer with numerous mitotic figures and multinucleated large cells. One- and 3-day NNK-treated cells grown in complete duct medium produced well-differentiated, mucinous tumours after their injection in nude mice. Analysis of DNA from these tumours for K-*ras* mutation at codons 12, 13 and 61 showed a G→A transition at codon 12 of the K-*ras* oncogene in tumour cells after 1 and 3 days of NNK treatment (Baskaran *et al*., 1994).

In-vivo treatment with cumulative doses of 150 and 300 mg/kg bw NNK produced significant increases in transformation frequency of tracheal cells in three of three and two of three rats, respectively (Zhu *et al.*, 1991).

Yoo *et al.* (2000) showed that normal human gingival keratinocytes immortalized with human papillomavirus 16 (IHGK) were transformed by NNK to IHGKN cells. Transformation of IHGK cells resulted in the activation of vascular endothelial growth factor associated with angiogenesis. Inactivation of the G1 phase of cell-cycle regulation occurred during immortalization before cell transformation, and was sustained after carcinogen exposure.

Other effects

In cultured human oral epithelial cells, treatment with NNK resulted in increased longevity and a sustained differentiated phenotype for 8.5–10 weeks. The treated cells displayed focal growth and morphological changes suggestive of early stages of cell transformation as compared with controls in which cells were terminally differentiated (Murrah *et al*., 1993).

A higher frequency of hyperplasia with hyperkeratosis was observed in the forestomach of hamster treated with a combination of nicotine and NNK as compared with either NNK or nicotine treatment alone. Squamous-cell papillomas were evident in the forestomach of animals treated with both NNK and nicotine (Chen *et al*., 1994).

NNK was shown to inhibit the production of IL-12 and TNF, key molecules of immune response in rat alveolar macrophages and stimulate the production of IL-10 and prostaglandin E_2 (Therriault *et al.*, 2003). Using model compounds NNKOAc and *N*-nitro-(acetoxymethyl)methylamine (NDMAOAc), it has been demonstrated that the above effect of NNK is mediated by α-methyl hydroxylation of NNK, the same pathway that induces DNA pyridyloxobutylation (Proulx *et al.*, 2004).

(ii) *NNAL*

NNAL was mutagenic in *S. typhimurium* TA1535 in the presence of a liver metabolic activation system from Aroclor-1254-induced rats or hamsters in the range of 0.025–0.2 μmol/plate [5.5–42 μg/plate] (maximum tested dose) (Brown *et al*., 2001b).

(*b*) *NNN*

(i) *Mutagenic and cytogenetic effects, DNA damage* (for details and references, see Table 18)

NNN was mutagenic in *S. typhimurium* strain TA100 in three of four assays (two with and one without exogenous metabolic activation), in strain TA1535 (with activation) and in TA1530 and TA7004 (without activation). NNN was also mutagenic in *Salmonella* tester strains YG7108 that carry the human *CYPs 2A6*, *1A1*, *3A4* and *3A5* in the absence of an exogenous activation system, but was not mutagenic in YG7108 itself or in YG7108 that carries *CYP2E1* (Kushida *et al.*, 2000a; Fujita & Kamataki, 2001). NNN was not mutagenic in *Salmonella* strains TA98 or TA1538 (single tests only).

NNN was a direct-acting mutagen in the Mutatox test using dark mutant M-169 of *Vibrio fischeri*, but was not mutagenic in this assay in the presence of a metabolic activation system from rat or hamster (Yim & Hee, 2001).

Using model compounds, it was shown that the putative diazohydroxide formed by 2′-hydroxylation of NNN has higher inherent mutagenicity toward *S. typhimurium* than the corresponding diazohydroxide formed by 5′-hydroxylation (Hecht & Lin, 1986).

NNN induced DNA strand breaks in primary rat hepatocytes but not in lung cells or alveolar macrophages of rabbits.

Table 18. Genetic and related effects of N′-nitrosonornicotine (NNN)

Test system	Result[a]		Dose[b] (LED or HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Salmonella typhimurium TA100, reverse mutation	–	+	2.5 µmol/plate [443 µg/plate]	Bartsch *et al.* (1980)
Salmonella typhimurium TA100, reverse mutation	–	+	250 µg/plate	Padma *et al.* (1989b)
Salmonella typhimurium, TA100, TA7004, reverse mutation	+	–	2000 µg/plate	Yim & Hee (2001)
Salmonella typhimurium TA100, TA7004, reverse mutation	+	–	500 µg/plate	Yim & Hee (2001)
Salmonella typhimurium TA1530, reverse mutation	+	NT	1000 µg/plate	Andrews *et al.* (1978)
Salmonella typhimurium TA1535, reverse mutation	–	+	1000 µg/plate	Padma *et al.* (1989b)
Salmonella typhimurium TA1538, TA 98, reverse mutation	–	–	1000 µg/plate	Padma *et al.* (1989b)
Salmonella typhimurium YG7108, reverse mutation	–	NT	0.7 mM [124 µg/mL]	Kushida *et al.* (2000a); Fujita & Kamataki (2001)
Salmonella typhimurium YG7108 -2A6/OR[c], reverse mutation	+	NT	0.2 mM [35.5 µg/mL]	Kushida *et al.* (2000a); Fujita & Kamataki (2001)
Salmonella typhimurium YG7108 -2E1/OR[d], reverse mutation	–	NT	0.7 mM [124 µg/mL]	Kushida *et al.* (2000a); Fujita & Kamataki (2001)
DNA strand breaks, primary rat hepatocytes *in vitro*	+	NT	5 mM [886 µg/mL]	Liu *et al.* (1990)
DNA strand breaks, isolated rabbit lung cells (Type II and Clara) and alveolar macrophages *in vitro*	–	NT	3 mM [531 µg/mL]	Becher *et al.* (1993)
DNA strand breaks, rat hepatocytes *in vitro*	–	NT	25 mM [4430 µg/mL]	Pool-Zobel *et al.* (1992)
Unscheduled DNA synthesis, freshly isolated rat hepatocytes *in vitro*	+	NT	1 mM [177 µg/mL]	Williams & Laspia (1979)
Unscheduled DNA synthesis, rabbit lung cells *in vitro*	–	NT	2 mM [354 µg/mL]	Dahl *et al.* (1990)
Gene mutation, Chinese hamster V79 cells, Hprt locus, *in vitro*	–	+	10 mM [1770 µg/mL]	Swedmark *et al.* (1994)
DNA strand breaks, human MRC-5 fetal lung cells *in vitro*	+	NT	5 mM [886 µg/mL]	Weitberg & Corvese (1993, 1997)
Sister chromatid exchange, human peripheral blood lymphocytes *in vitro*	–	NT	100 µg/mL	Padma *et al.* (1989b)

Table 18 (contd)

Test system	Result[a]		Dose[b] (LED or HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Chromosome aberrations, human peripheral blood lymphocyte *in vitro*	–	NT	100 µg/mL	Padma *et al.* (1989b)
DNA strand breaks, Sprague-Dawley rat liver *in vivo*	+		100 mg/kg bw po × 1 [565 µmol/kg bw]	Pool-Zobel *et al.* (1992)
Micronucleus formation, Swiss male mice bone marrow *in vivo*	+		250 mg/kg bw ip ×2	Padma *et al.* (1989b)

[a] +, positive;–, negative; NT, not tested
[b] LED, lowest effective dose; HID, highest ineffective dose; ip, intraperitoneal injection
[c] Co-expressing human CYP 2A6 together with human NADPH-cytochrome P450 reductase (OR)
[d] Co-expressing human CYP 2E1 together with human NADPH-cytochrome P450 reductase (OR)

NNN induced DNA strand breaks in MRC-5 human fetal lung cells. When these MRC-5 cells were treated with NNN in combination with enzymatically generated oxygen radicals, DNA strand-breakage increased by approximately 50%, while oxygen-radical scavengers (superoxide dismutase, catalase, mannitol) significantly reduced the DNA damage caused by NNN (Weitberg & Corvese, 1993).

NNN induced DNA single-strand breaks in cultured primary rat hepatocytes (as measured by the alkaline elution assay). It did not cause any marked DNA damage (as measured by the alkaline elution assay) in isolated Clara and type II cells from rabbit lung and in isolated rabbit alveolar macrophages. NNN induced unscheduled DNA synthesis in freshly isolated hepatocytes from adult rats. NNN had genotoxic effects in primary rat hepatocytes *in vitro*, measured as DNA damage by alkaline elution and nick translation (Pool-Zobel *et al.*, 1992). NNN-induced mutations were not observed at the *Hprt* locus of V79 Chinese hamster cells after S9 metabolic activation in a co-cultivation system that used either freshly isolated rat hepatocytes or H4IIE rat hepatoma cells (Swedmark *et al.*, 1994).

NNN was mutagenic in a mixture of pooled oral tissues (gingiva, buccal cavity, pharynx and sublingua), and in tongue and oesophageal tissue in *lacZ*-transgenic mice (Muta^{TM}Mouse) (von Pressentin *et al*., 1999).

NNN did not induce cytogenetic effects (sister chromatid exchange or chromosomal aberrations) in human peripheral blood lymphocytes.

NNN had genotoxic effects in Sprague-Dawley rat liver *in* vivo, measured as DNA damage by alkaline elution and nick translation (Pool-Zobel *et al*., 1992). *In vivo*, NNN induced micronuclei in the bone marrow of Swiss mice.

In animal-mediated DNA-repair assays with *Escherichia coli* K-12 strains (injected intravenously just before nitrosamine treatment), intraperitoneal administration of NNN to mice caused dose-dependent genotoxic effects in indicator bacteria recovered from various organs of the treated animals. The genotoxic effect was enhanced by ethanol treatment prior to carcinogen treatment (Knasmüller *et al*., 1994).

(ii) *Other effects*

In cultured human oral epithelial cells, treatment with NNN resulted in increased longevity and a sustained differentiated phenotype for 8.5–10 weeks. The treated cells displayed focal growth and morphological changes that were suggestive of early stages of cell transformation in comparison with control cells which were terminally differentiated (Murrah *et al*., 1993).

Hamster cheek-pouch epithelium showed histological changes, including hyperplasia, hyperkeratosis and, in one animal, moderate dysplasia, when treated with nicotine combined with NNN. These changes were more frequent than after treatment with NNN or nicotine alone (Chen *et al*., 1994).

Exposure of Syrian hamster buccal mucosa to NNN, five times per week for 24 weeks, did not result in clinical or histological changes (Papageorge *et al*., 1996).

(*c*) *NAB*

Using genetically engineered *S. typhimurium* strain YG7108 that overexpresses human CYP, NAB induced mutation in the strains that contain CYP3A4, CYP2A6, CYP1A1 or CYP3A5. CYP3A4-carrying constructs induced the greatest mutagenicity (0.071 revertants/nmol NAB/pmol CYP) (Fujita & Kamataki, 2001).

(*d*) *NAT*

Using genetically engineered *S. typhimurium* strain YG7108 that overexpresses human CYP, NAT induced mutation in the strain that contains CYP2A6 (0.164 revertants/nmol NAB/pmol CYP) (Fujita & Kamataki, 2001).

4.5 Mechanistic considerations

4.5.1 *4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)*

The formation of DNA adducts is pivotal in the carcinogenic process (Miller, 1994). If not repaired, DNA adducts cause permanent mutations due to miscoding events during the replication of adducted DNA (Singer & Essigmann, 1991; Seo *et al.*, 2000). When these mutations occur in critical regions of growth control genes, such as oncogenes and tumour-suppressor genes, cancer can result. The occurrence of multiple mutated genes in tumours that are caused by tobacco products is consistent with the extensive range of DNA damage that is caused by metabolically activated tobacco carcinogens (Hecht, 2003). However, NNK requires metabolic activation, generally through catalysis by CYP enzymes, before this genotoxic mechanism of cancer causation can occur. Extensive studies in laboratory animals have clearly demonstrated that the pathway of metabolic activation → persistent DNA adducts → mutations is critical to the carcinogenesis of NNK in the lung and nasal cavity. Fewer experimental studies have examined the genotoxic mechanism of the carcinogenicity of NNK in oral and pancreatic cells and tissues. Studies that used human cells and tissues have investigated potential parallels between experimental and human systems. Other mechanisms that contribute to the carcinogenesis of NNK have also emerged in recent years. This section examines the genotoxic and other mechanisms of the carcinogenesis of NNK, and focuses on comparisons between experimental and human systems.

(*a*) *Genotoxic mechanisms*

(i) *Metabolism*

A common metabolic event in virtually all systems (rodent and human) is the conversion of NNK to NNAL, during which (*S*)-NNAL predominates (Hecht, 1998, 2002). Studies in rats have demonstrated that (*S*)-NNAL is extensively distributed in the body and accumulates and persists in the lung, possibly at a receptor site (Wu *et al.*, 2002; Zimmerman *et al.*, 2004). (*S*)-NNAL is efficiently reconverted to NNK in rats (Zimmerman *et al.*, 2004). (*S*)-NNAL also persists in humans as shown by its slow excretion relative to that of (*R*)-NNAL

after cessation of tobacco use (Hecht *et al.*, 2002). Rat CYP2A3 and human CYP2A13 have similarly high catalytic efficiencies for the reconversion of (*S*)-NNAL to NNK (Tables 11 and 16; Jalas *et al.*, 2003a, 2005). The accumulation of (*S*)-NNAL in the lung may be a critical feature of the selectivity of NNK for induction of lung tumours in rodents. NNAL and its glucuronides are excreted in the urine of rodents, primates and humans (Hecht, 1998, 2002).

Rodent lung, oral mucosa, nasal mucosa and liver all metabolize NNK by α-hydroxylation at its methylene and methyl carbons to produce intermediates that bind to DNA (Hecht, 1998). These reactions are catalysed by CYP enzymes. Steady-state kinetic parameters for CYP-catalysed NNK metabolism have been reported for five rat enzymes, two mouse enzymes, two rabbit enzymes and eight human enzymes (Tables 11 and 14; Jalas *et al.*, 2005). Of these, rat CYP2A3, mouse CYP2A5, rabbit CYPs 2A10/11 and human CYPs 2A13 and 2B6 exhibit the lowest K_m values and may be the most important catalysts of NNK bioactivation in the respective species (Table 14). Members of the CYP2A subfamily appear to be the best catalysts of NNK α-hydroxylation across species, and CYP2A13, which has catalytic properties for NNK metabolism that are very similar to those of rat CYP2A3, may be particularly important in the bioactivation of NNK by the human lung (Jalas *et al.*, 2005). Rat pancreatic microsomes converted NNK to NNAL, (NNK)ADP$^+$ and (NNK)ADPH, and converted NNAL to (NNAL)ADP$^+$; products of α-hydroxylation were not observed (Peterson *et al.*, 1994).

NNK and/or NNAL are metabolically activated by a variety of human tissues and cells including those from the oral cavity, lung, oesophagus, cervix, urinary bladder and liver (Hecht, 1998; Prokopczyk *et al.*, 2001; Vondracek *et al.*, 2001). The extents of metabolism by α-hydroxylation are generally lesser than those observed in rodents. NNK and NNAL have been detected in human pancreatic juice (Prokopczyk *et al.*, 2002). Human pancreatic microsomes converted NNK to NNAL, but no products of α-hydroxylation of NNK or NNAL were observed (Anderson *et al.*, 1997).

(ii) *DNA adducts*

Methyl and pyridyloxobutyl DNA adducts of NNK have been characterized *in vitro* and *in vivo* (Hecht, 1998; Wang *et al.*, 2003; Hecht *et al.*, 2004c). Extensive studies have examined the mechanisms of lung tumour formation in A/J mice treated with a single dose of NNK. A consistent body of evidence including structure–activity studies, investigations of deuterated NNK analogues, analysis of the occurrence and persistence of DNA adducts and effects on the AGT-repair enzyme strongly implicates *O*^{6}-MeGua as the critical DNA adduct in lung tumour induction by NNK in this mouse strain (Peterson & Hecht, 1991; Hecht, 1998; Peterson *et al.*, 2001; Jalas & Hecht, 2003; Jalas *et al.*, 2003b; Thomson *et al.*, 2003). *O*^{6}-MeGua is known to have miscoding properties that cause G→A transitions (Loechler *et al.*, 1984). Mutations in the K-*ras* gene in A/J mouse lung tumours induced by NNK are predominantly G→A transitions, which is consistent with the important role of *O*^{6}-MeGua (Hecht, 1998). A different picture emerges from studies of mechanisms of NNK-induced lung and nasal cavity carcinogenesis in Fischer 344 rats. Strong

evidence, based on structure–activity considerations and extensive studies of the formation and persistence of DNA adducts in individual cell types of the lung and different regions of the nasal mucosa and the effects of inhibitors of NNK carcinogenesis, indicate that a combination of O^6-MeGua and HPB-releasing pyridyloxobutyl DNA adducts is important in lung carcinogenesis and that these latter adducts are critical in nasal cavity carcinogenesis by NNK (Hecht, 1998). Fewer studies have been carried out in oral tissue. However, it is known that rat oral tissue actively metabolizes NNK by all known pathways including α-hydroxylation, and 7-MeGua has been identified in these tissues (Murphy *et al.*, 1990a).

Methyl and pyridyloxobutyl DNA adducts have been identified in the lungs of smokers (Hecht, 1998). While the methyl adducts, 7-MeGua and O^6-MeGua, may have multiple sources and are also found in nonsmokers, only NNK and NNN are probable precursors to pyridyloxobutyl DNA adducts. Cellular binding has also been observed in human oral keratinocyte cell lines exposed to NNK (Vondracek *et al.*, 2001).

(iii) *Mutations*

Mutations in the K-*ras* gene are frequently found in A/J mouse and hamster lung tumours induced by NNK (Hecht, 1998). The most common mutation is a GGT→GAT transition in codon 12 of the K-*ras* gene. In-vitro studies have demonstrated that 7-MeGua, O^6-MeGua and O^6(POB-1-yl)Gua are preferentially formed at the second G of codon 12 of the K-*ras* gene (Ziegel *et al.*, 2003). Both O^6-MeGua and O^6(POB-1-yl)Gua are known to cause predominantly G→A transition mutations (Loechler *et al.*, 1984; Pauly *et al.*, 2002). The pyridyloxobutylating agent, NNKOAc, causes GGT→TGT and GGT→GTT mutations in codon 12 in addition to GGT→GAT mutations (Ronai *et al.*, 1993). Although mutations in K-*ras* in lung tumours from mice and hamsters are consistent with the properties of the DNA adducts formed by NNK, other factors are also involved. For example, only two of 22 lung tumours induced by NNK in relatively insensitive C57BL/6 mice had K-*ras* mutations (Devereux *et al.*, 1993). In another study, treatment of mice with NNK followed by butylated hydroxytoluene increased lung tumour induction compared with NNK alone, but decreased the frequency of GGT→GAT mutations in codon 12 of K-*ras* (Matzinger *et al.*, 1994). The frequency of activation of K-*ras* and GGT→GAT mutation in codon 12 is not affected by the time after NNK treatment, nor are the proliferative activity of the lung lesions and the presence of mutations correlated. Thus, K-*ras* gene mutations appear to play a minor role in the selective growth advantage of NNK-induced lung lesions in A/J mice (Kawano *et al.*, 1996). In the Muta Mouse treated with NNK, A:T→T:A and A:T→C:G transversions were the major mutations observed in the lung and liver *cII* genes; G:C→A:T transitions were also observed, but to a lesser extent (Hashimoto *et al.*, 2004). There is no evidence of K-*ras* or *p53* mutations in lung tumours induced by NNK in rats (Hecht, 1998). No mutations have been detected in the *p53* gene from NNK-induced mouse lung tumours and only one of 24 hamster lung tumours examined had a mutation in the *p53* gene (Oreffo *et al.*, 1993; Hecht, 1998).

Mutations in codon 12 of the K-*RAS* gene are present in 24–50% of human primary lung adenocarcinomas, but are rarely seen in other types of lung tumour (Rodenhuis & Slebos, 1992; Mills *et al.*, 1995; Westra *et al.*, 1996). These mutations are more common in smokers and former smokers than in nonsmokers, which suggests that they may be induced by a component of tobacco smoke (Westra *et al.*, 1993). The most frequently observed mutation is GGT→TGT, which typically represents about 60% of the mutations in codon 12, followed by GGT→GAT (20%) and GGT→GTT (15%). The prevalence of G→T mutations has led to speculation that they may be due to polycyclic aromatic hydrocarbons, which can induce such mutations through the diol epoxide metabolic activation pathway (You *et al.*, 1989; Westra *et al.*, 1993). However, G→T mutations are also induced by NNKOAc (Ronai *et al.*, 1993). In A/J mice, the O^6-MeGua pathway of NNK metabolic activation is clearly the major pathway involved in tumour induction, which is consistent with the high percentage of GGT→GAT mutations in the K-*ras* gene isolated from mouse lung tumours induced by NNK. However, in Fischer 344 rats, both the pyridyloxobutylation and methylation pathways are critical in the lung tumorigenesis of NNK. The relative importance of these pathways in human lung carcinogenesis is not known. If pyridyloxobutylation is critical, as in the rat, a higher percentage of G→T transversions than that observed in mice would be expected as a result of exposure to NNK. In the absence of additional information, it is difficult to assign mutations in human genes to particular carcinogen adducts for an exposure that is as complex as that to tobacco smoke. Numerous compounds that damage DNA are present in tobacco smoke and many of these cause G→T transversion mutations: examples, in addition to nitrosamines and polycyclic aromatic hydrocarbons, include aromatic amines, oxygen radicals and α,β-unsaturated aldehydes (Singer & Essigmann, 1991; Moriya, 1993; Moriya *et al.*, 1994; Nesnow *et al.*, 1995). With respect to the *p53* gene, which is commonly mutated in tobacco-related cancers, mutations at G are frequently observed, which is consistent with the multiple carcinogens in tobacco products that bind to G. The spectrum of mutations in the *p53* gene from lung tumours has been attributed in part to reactions with polycyclic aromatic hydrocarbon diol epoxides, subject to the limitations discussed above; at present, no evidence has been found that NNK produces a similar mutational spectrum (Pfeifer *et al.*, 2002; Ziegel *et al.*, 2004).

(*b*) *Other mechanisms*

NNK is a high-affinity agonist for both the β_1- and β_2-adrenergic receptors in human pulmonary adenocarcinoma cell lines and in Chinese hamster ovary cell lines that have been transfected with the human β_1- or β_2-adrenergic-receptor gene (Schuller, 2002). NNK and other β-adrenergic receptor agonists stimulate the release of arachidonic acid from cell membrane phospholipids, which results in stimulated DNA synthesis and proliferation of human pulmonary adenocarcinoma cells. The mitogenic response to NNK is reduced upon treatment with β-adrenergic receptor antagonists, such as propanolol, and with cyclooxygenase and lipoxygenase inhibitors. Similar results have been obtained in pancreatic cells. In cell lines derived from human pancreatic adenocarcinomas, NNK

stimulated the release of arachidonic acid, which led to DNA synthesis and cell proliferation. In a model of pancreatic carcinogenesis that was induced transplacentally by treatment of pregnant hamsters with NNK and ethanol, treatment of the offspring with ibuprofen and the 5-lipoxygenase-activating protein inhibitor MK886 began 4 weeks after the birth and inhibited pancreatic tumorigenesis (Schuller *et al.*, 2002). These results indicate that receptor binding of NNK could play a role in human lung and pancreatic carcinogenesis (Schuller, 2002).

NNK binds to the α_7 nicotinic acetylcholine receptor (α_7nAChR) in small-cell lung carcinoma and pulmonary neuroendocrine cells, which results in the influx of Ca^{2+}, release of 5-hydroxytryptamine (serotonin) and activation of a mitogenic pathway mediated by protein kinase C, Raf-1, mitogen-activated protein kinase and c-*Myc*. Unstimulated small-cell lung carcinoma cells from smokers demonstrated high base levels of 5-hydroxytryptamine release and individual downstream signalling components in comparison with pulmonary neuroendocrine cells. Subchronic exposure of the latter cells to NNK up-regulated the α_7nAChR and its associated mitogenic pathways (Schuller *et al.*, 2003). NNK at 0.1 nM simultaneously stimulates phosphorylation of the oncogenic proteins Bcl2 and c-Myc through the α_7nAChR in association with increased proliferation of human small-cell lung carcinoma cells, which suggests that NNK facilitates a functional cooperation between Bcl2 and c-Myc in a mechanism that involves phosphorylation of both regulators (Jin *et al.*, 2004).

The $p16^{INK4a}$ (*p16*) tumour-suppressor gene can be inactivated by hypermethylation of its promoter region. In Fischer 344 rats, 94% of adenocarcinomas induced by NNK were hypermethylated at the *p16* gene promoter and this change was frequently detected in precursor lesions of the tumours such as adenomas and hyperplasias. This timing of *p16* hypermethylation was reproduced in human squamous-cell carcinomas in which the *p16* gene was coordinately methylated in 75% of carcinoma *in situ* lesions adjacent to squamous-cell carcinomas that harboured this change. The frequency of *p16* hypermethylation increased during disease progression from basal-cell hyperplasia to squamous metaplasia to carcinoma *in situ* (Belinsky *et al.*, 1998). Hypermethylation of *p16* was also detected in 45% of rat liver tumours induced by NNK, and is a common event in human hepatocellular carcinoma (Pulling *et al.*, 2001). The death-associated protein kinase (*DAPK*) gene is methylated in 23–44% of human non-small-cell lung cancers and in 52% of mouse lung tumours induced by NNK (Pulling *et al.*, 2004). *DAPK* methylation was observed at a similar prevalence in NNK-induced hyperplasias and adenocarcinomas, which suggests that inactivation of this gene is one pathway for tumour development in the mouse lung (Pulling *et al.*, 2004). The retinoic acid receptor gene β (*RAR-β*), which encodes one of the primary receptors for retinoic acid, is down-regulated by methylation in human lung cancer (Vuillemenot *et al.*, 2004). Methylated alleles of this gene were detected in virtually all primary lung tumours induced in mice by NNK and in 54% of preneoplastic hyperplasias induced by NNK (Vuillemenot *et al.*, 2004).

The serine/threonine kinase Akt (or protein kinase B) is activated in non-small-cell lung cancer cells and promotes cellular survival and resistance to chemotherapy or radiation

(Brognard *et al.*, 2001). NNK activates Akt in non-immortalized human airway epithelial cells. Activation of Akt by NNK occurred dose dependently within minutes and depended upon α_7nAChR. Activated Akt increased phosphorylation of certain downstream substrates that control the cellular cell cycle and protein translation, and partially induced a transformed phenotype. Active Akt was detected in airway epithelial cells and lung tumours from NNK-treated A/J mice and in human lung cancers from smokers (West *et al.*, 2003). These studies were extended by examining Akt activation at intermediate steps in carcinogenesis. The phosphatidylinositol 3′-kinase/Akt pathway was analysed in isogenic, immortalized or tumorigenic human lung bronchial epithelial cells *in vitro* and in a spectrum of NNK-induced mouse lung lesions *in vivo*. Progressive activation of the phosphatidylinositol 3′-kinase/Akt pathway correlated with phenotypic progression of lung epithelial cells, which strengthens the hypothesis that Akt activity plays a role in lung tumorigenesis (West *et al.*, 2004).

4.5.2 N′-*Nitrosonornicotine (NNN)*

A convincing body of data demonstrates that NNN requires metabolic activation to form DNA adducts that drive the mutagenic and carcinogenic processes. Metabolic activation of NNN occurs by 2′-hydroxylation and 5′-hydroxylation and leads to the formation of diazohydroxides that bind to DNA and are mutagenic. This process is observed in both rodent and human tissues. Metabolic activation of NNN is catalysed by CYP enzymes and, among these, CYP2A enzymes are outstanding catalysts in both rodents and humans. The formation of HPB-releasing DNA adducts of NNN has been clearly demonstrated in the rat nasal mucosa and oesophagus, which are target tissues of NNN carcinogenesis. HPB-releasing haemoglobin adducts of NNN are also produced in rats. As discussed above, HPB-releasing DNA and haemoglobin adducts have been detected in humans exposed to tobacco products, and their sources could be either NNK or NNN or both. Thus, there are clear parallels between the mechanisms of metabolic activation of NNN in rodents and humans.

5. Summary of Data Reported and Evaluation

5.1 Exposure data

Tobacco-specific *N*-nitrosamines, including 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), *N*′-nitrosonornicotine (NNN), *N*′-nitrosoanabasine (NAB) and *N*′-nitrosoanatabine (NAT), occur widely in tobacco and tobacco smoke. They are formed by the nitrosation of nicotine and other tobacco alkaloids and have been detected in green tobacco leaves from *Nicotiana tabacum* and *N. rustica* species; however, the largest quantities of tobacco-specific *N*-nitrosamines are formed during tobacco curing and processing and additional amounts are formed during smoking. Tobacco-specific *N*-nitrosamines occur in all

commercially and non-commercially prepared tobacco products including cigarettes, cigars, *bidis*, pipe tobacco and smokeless tobacco products. *N*-Nitrosamines occur in a wide variety of both food and non-food products, but the amounts of tobacco-specific *N*-nitrosamines in all tobacco products exceed the levels of other *N*-nitrosamines in other commercial products by several orders of magnitude. The highest levels of tobacco-specific *N*-nitrosamines are measured in smokeless tobacco products. For example, levels of NNK up to 17.8 μg/g have been measured in North American and European smokeless tobacco products; up to 245 μg/g have been measured in products used in India; and up to 7870 μg/g have been measured in Sudanese *toombak*. Levels of NNN up to 135 μg/g have been measured in North American and European smokeless tobacco products; up to 1356 μg/g have been measured in products used in India; and up to 3085 μg/g have been measured in Sudanese *toombak*. These compounds are also present in secondhand tobacco smoke. The degree of exposure to tobacco-specific *N*-nitrosamines depends not only on the levels of these compounds in tobacco products or smoke, but also on the manner in which the products are used.

5.2 Human carcinogenicity data

No data were available to the Working Group.

5.3 Animal carcinogenicity data

NNK

In numerous studies in mice, NNK induced lung adenomas independent of the route of administration.

In studies by subcutaneous injection, benign and malignant tumours of the lung, nasal cavity and liver were induced in rats. In two of four experiments in hamsters, lung adenomas and adenocarcinomas or adenosquamous carcinomas were induced in males and females. In the two other experiments, adenomas were observed. Nasal cavity tumours involving the forebrain were observed in a limited study in mink.

In a study by administration in the drinking-water and another by oral swabbing, combined benign and malignant lung tumours (adenoma, adenosquamous carcinoma and carcinoma) were induced in male rats. In the drinking-water study, NNK produced benign and malignant pancreatic tumours. In the oral swabbing study, combined benign and malignant tumours of the liver and nasal cavity were observed. A significant increase in the incidence of liver and lung tumours was reported in female rats when NNK was instilled into the urinary bladder.

In two studies, the offspring of mice were exposed transplacentally by intraperitoneal injection of the dams. Liver tumours were observed in male offspring in both studies and in female offspring in one study. In one of these studies, lung tumours were also observed in male offspring.

In studies of the offspring of hamsters given NNK during pregnancy, intratracheal instillation of the dams resulted in adenocarcinomas of the nasal cavity in male offspring and adrenal pheochromocytomas in male and female offspring in one study. In a second study, subcutaneous injection of NNK into dams induced respiratory tract (nasal cavity, larynx and trachea) tumours in male and female offspring. When dams were injected subcutanously or treated by intratracheal instillation, nasal cavity and adrenal gland tumours developed in male and female offspring in a third study.

Intraperitoneal administration of NNK-*N*-oxide induced lung adenomas in female mice.

In an oral swabbing study, NNK in combination with NNN increased the incidence of oral tumours in rats.

4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL)

In one study in male rats in which NNAL, a principal metabolite of NNK, was administered in the drinking-water, adenomas, adenocarcinomas and adenosquamous carcinomas of the lung and benign and malignant pancreatic tumours were induced.

In three studies in female mice, intraperitoneal injection of NNAL induced lung adenomas. In one of these studies, adenocarcinomas were also observed.

NNN

In four studies in female mice, intraperitoneal injection of NNN produced lung adenomas. In another study in mice, lung adenomas were induced by intraperitoneal injection of NNN in males and females.

In two studies in rats in which NNN was given in the drinking-water and one study in which it was added to a liquid diet, benign and malignant oesophageal tumours were observed in males and females. Benign nasal cavity tumours were also observed in rats treated through the liquid diet.

In rats, subcutaneous injection of NNN induced malignant or benign (combined) and malignant nasal cavity tumours in males and females in two studies. In one study in hamsters, subcutaneous injection of NNN induced tracheal tumours in males and females and benign and malignant tumours of the nasal cavity in males. In two limited studies in mink treated with NNN by subcutaneous injection, nasal cavity tumours that invaded the forebrain were observed in females.

Skin application of NNN in female mice induced a non-significant increase in the incidence of skin papillomas and carcinomas.

Metabolites of NNN (3′-hydroxy-NNN, 4′-hydroxy-NNN or NNN-1-*N*-oxide) were tested by intraperitoneal injection into female mice and resulted in the induction of lung adenomas in mice exposed to 4′-hydroxy-NNN. Administration of NNN-1-*N*-oxide in the drinking-water increased the incidence of benign and malignant oesophageal tumours in male and female rats in one study and that of colon tumours in female hamsters in another study.

In one oral swabbing study, NNN in combination with NNK increased the incidence of oral tumours in rats.

NAB

In one study in female mice, intraperitoneal injection of NAB induced lung adenomas.

In one study in rats in which NAB was administered in the drinking-water, oesophageal carcinomas and/or papillomas were induced in males and females. Another study in male rats gave negative results.

Subcutaneous injection of NAB into hamsters gave negative results in one study.

NAT

Subcutaneous injection of NAT into male and female rats did not induce tumours at any site.

5.4 Other relevant data

NNK and its metabolite NNAL

Extensive studies have examined the metabolism of NNK and the formation of DNA adducts by NNK and its metabolite NNAL in humans and laboratory animals; the metabolic pathways and structures of DNA adducts have been characterized comprehensively. NNK and NNAL have been detected in the saliva of smokeless tobacco users, and NNAL and another metabolite of NNK, NNAL-glucuronide, have been quantified in human urine. The presence of these metabolites, which are specific to exposure to tobacco products (e.g. in smokers, users of smokeless tobacco and nonsmokers exposed to secondhand tobacco smoke), signals human uptake and metabolism of NNK, and their quantification allows an estimation of the dose of NNK absorbed. Dose calculations show that the total amounts of NNK taken up by people who used tobacco products for a period of 30 years or more approximate the total amounts that induce tumours in rats.

The metabolic activation of NNK and NNAL to DNA adducts is critical for the expression of their carcinogenic activities. The metabolic activation process has been documented extensively in laboratory animals. Cytochrome P450 enzymes are the principal catalysts of this process, and those in the 2A family appear to be the most efficient in both humans and laboratory animals. Macromolecular adducts formed after the metabolic activation of NNK and/or NNN have been detected in smokers, in smokeless tobacco users and in laboratory animals treated with these carcinogens. In laboratory animals, persistence of these adducts is associated with tumour formation.

NNK is a genotoxic compound. It was shown to be mutagenic in bacteria, in rodent fibroblasts and in human lymphoblastoid cells *in vitro*. It caused cytogenic effects in a variety of mammalian cells *in vitro* and induced transformation of the pancreatic duct cells of hamsters. *In vivo,* NNK induced micronucleus formation in the bone marrow of

mice and DNA strand breaks in the hepatocytes of rats and hamsters. NNAL was reported to be mutagenic in *Salmonella* in a single study.

In addition to the classical mechanisms of carcinogenesis that proceed through the formation of DNA adducts, NNK also binds to nicotinic and other receptors, which leads to downstream effects that contribute to the development of cancer. These effects have been observed in experimental systems including pancreatic and lung cells from humans and laboratory animals.

NNN

The major route of metabolic activation to DNA adducts is α-hydroxylation adjacent to the nitroso group, which is mediated principally by cytochrome P450 enzymes and, in particular, by those of the 2A family. The human oesophagus catalyses α-hydroxylation of NNN, and this process is especially efficient in the rat oesophagus and nasal mucosa, which are target tissues for the carcinogenicity of NNN. NNN has been detected in the saliva of smokeless tobacco users. The uptake and metabolism of NNN by smokers and smokeless tobacco users has been demonstrated by its quantitation and that of its glucuronide in human urine. The metabolic pathways of NNN have been characterized extensively in laboratory animals and there are distinct parallels can be seen between the metabolism of NNN in humans and laboratory animals.

NNN is a genotoxic compound. It was shown to be mutagenic in bacteria, but not in mammalian cells *in vitro*. NNN induced DNA strand breaks in human fetal lung cells and in primary rat hepatocytes *in vitro*. It did not show cytogenetic activity *in vitro*, but induced micronuclei in the bone marrow of mice *in vivo* in a single study.

NAB

NAB has been detected in the saliva of *toombak* users and has been quantified together with its glucuronide in the urine of smokers and smokeless tobacco users. The metabolism of NAB by α-hydroxylation and other pathways has been characterized in rats.

The genotoxicity of NAB has not been tested extensively. It was shown to be mutagenic in various strains of *Salmonella typhimurium*, each of which co-expressed a different form of human cytochrome P450 enzyme.

NAT

NAT has been detected in the saliva of smokeless tobacco users, and has been quantified together with its glucuronide in the urine of smokers and smokeless tobacco users.

The genotoxicity of NAT has not been tested extensively. It was shown to be mutagenic in a strain of *S. typhimurium* that expressed human cytochrome P450 2A6.

5.5 Evaluation

There is *inadequate evidence* in humans for the carcinogenicity of tobacco-specific *N*-nitrosamines.

There is *sufficient evidence* in experimental animals for the carcinogenicity of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and its metabolite 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL).

There is *sufficient evidence* in experimental animals for the carcinogenicity of *N*′-nitrosonornicotine (NNN).

There is *limited evidence* in experimental animals for the carcinogenicity of *N*′-nitrosoanabasine (NAB).

There is *inadequate evidence* in experimental animals for the carcinogenicity of *N*′-nitrosoanatabine (NAT).

Overall evaluation

4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and *N*′-nitrosonornicotine (NNN) are *carcinogenic to humans (Group 1).*

N′-Nitrosoanabasine (NAB) is *not classifiable as to its carcinogenicity to humans (Group 3).*

N′-Nitrosoanatabine (NAT) is *not classifiable as to its carcinogenicity to humans (Group 3).*

In making the overall evaluation of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and *N*′-nitrosonornicotine, the Working Group took into consideration the following mechanistic evidence (detailed in Section 5.4).

4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone and *N*′-nitrosonornicotine are the most abundant strong carcinogens in smokeless tobacco; uptake and metabolic activation in smokeless tobacco users have been clearly observed. In rats, combined application of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and *N*′-nitrosonornicotine induced oral tumours consistent with their induction by smokeless tobacco. One of the mechanisms of carcinogenicity is cytochrome P450-mediated α-hydroxylation, which leads to the formation of DNA and haemoglobin adducts that are commonly detected in users of tobacco.

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