

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
<i>NNK</i>										
Mouse	x					x		x		x
Rat	x	x	x ^a				x	x		
Hamster	x	x		x			x ^c		x ^c	
Mink	x	(x)								
<i>NNN</i>										
Mouse	x					x				
Rat		x	x ^b		x					
Hamster		x		x						
Mink		(x)								

NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNN, *N'*-nitrosomonicotine

^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 μ mol/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 [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 μ mol/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 intravesicularly 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 μ mol. 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-Wallis 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/m³) 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 μ mol and 50 μ mol 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 μ mol NNK and 20 mice injected with 20 μ mol 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 μ mol 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:SEN CAR BR mice, 50–55 days of age, received topical applications of 2.8 $\mu\text{mol}/\text{mouse}$ NNAL in 100 μL acetone every other day (10 doses; total dose, 28 $\mu\text{mol}/\text{mouse}$). 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'*-Nitrosornicotine (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 NNN 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 μ mol/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 μ mol or 200 μ mol 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 μ mol NNK and 20 mice injected with 20 μ mol 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 μ mol, 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 $\mu\text{g}/\text{mouse}$ 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 μ g) 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 μ mol 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) μ mol 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).