PART 1.

CONCORDANCE BETWEEN CANCER IN HUMANS AND IN EXPERIMENTAL ANIMALS

CHAPTER 4.

Smokeless tobacco and its constituents

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Introduction

Smokeless tobacco is defined as follows in Volume 89 of the *IARC Monographs* (IARC, 2007): "Smokeless tobacco is used without burning the product, and can be used orally or nasally. Oral smokeless tobacco products are placed in the mouth, against the cheek or behind the lip and sucked (dipped) or chewed. Tobacco pastes or powders are used in a similar manner and applied to the gums or teeth. Fine tobacco mixtures are usually inhaled and absorbed in the nasal passages."

This chapter considers carcinogenicity studies, data on constituents, and mechanistic investigations on smokeless tobacco, to evaluate overall coherence between observations in humans and in experimental animals. Because of the differences between human use of smokeless tobacco and the exposure conditions in studies in experimental animals, the term "coherence", which means *logical consistency*, is more appropriate here than the term "concordance", which connotes a one-to-one agreement, with no conflicting data.

Coherence: carcinogenicity of smokeless tobacco in humans versus experimental animals

Evaluations of smokeless tobacco use by the *IARC Monographs* concluded that this practice is *carcinogenic to humans* (Group 1), causing cancers of the oral cavity, oesophagus, and pancreas (IARC, 1985, 2007, 2012). A meta-analysis of epidemiological data also concluded that use of smokeless tobacco significantly increased the risk of these cancers (Boffetta et al., 2008). A recent population-based case–control study, which was carried out in New England, USA, and was not included in the above-mentioned evaluations, demonstrated a statistically significant association between ever use of smokeless tobacco and the risk of head and neck squamous cell carcinoma (including cancers of the oral cavity, larynx, and pharynx) (Zhou et al., 2013). This section considers coherence between these conclusions and studies of the carcinogenicity of smokeless tobacco in experimental animals.

The use of smokeless tobacco by humans is a voluntary practice engaged in by hundreds of millions of people worldwide. There are great variations in use of smokeless tobacco: in Sweden, fine-cut tobacco, called *snus*, is placed between the upper lip and teeth; in North America, fine-cut tobacco, frequently in teabag-like sachets, is placed between the cheek and gums; and in South-East Asia and other parts of the world, there are vast arrays of different practices (IARC, 2007). Processed and fermented tobacco of varying types and blends are the common ingredients in all of these practices. Nicotine, perhaps along with other tobacco alkaloids and constituents, is the addictive substance

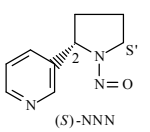
that drives the continuing use of these products (DHHS, 1988; Stolerman and Jarvis, 1995; Benowitz, 1999; IARC, 2007).

With reference to the use of smokeless tobacco by humans, it has not yet been possible to develop an experimental model in which laboratory animals *voluntarily and habitually* consume these products the way they are used by humans. Various approaches have been explored, including addition of tobacco to the diet, oral treatment of animals with tobacco extracts, exposure of animals to powdered tobacco by inhalation, placement of tobacco in the cheek pouch of hamsters, and surgical modification of the oral cavity. However, none of these methods faithfully replicate the human habit, and they have not always produced statistically significant results in carcinogenicity studies. The most consistent findings in animal carcinogenicity studies of smokeless tobacco have been reported in a model in which an artificial lip canal is created by surgery on rats. Several studies of this type produced tumours of the oral cavity, including squamous cell carcinomas, and their incidence was significantly increased compared with controls in some experiments (IARC, 2007). Also, in one study, insertion of snuff into the cheek pouch of hamsters infected with herpes simplex virus type 1 (HSV-1) or type 2 (HSV-2) significantly increased the incidence of squamous cell carcinoma compared with that in animals infected with HSV-1 or HSV-2 and not administered snuff (Park et al., 1986). This observation has not been replicated, and its relevance to human use of smokeless tobacco is unclear. Overall, there is some coherence between studies in experimental animals on the carcinogenicity of smokeless tobacco and cancer of the oral cavity in humans as induced by use of smokeless tobacco; the conclusion of the *IARC Monographs* that there is *sufficient evidence* in experimental animals for the carcinogenicity of smokeless tobacco followed from this evidence (IARC, 2012). However, the results are somewhat inconsistent and are limited by the requirement of surgery and other unnatural approaches in an attempt to replicate in laboratory animals the voluntary use of smokeless tobacco by humans.

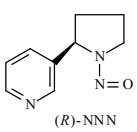
Coherence: carcinogenicity of smokeless tobacco in humans versus carcinogenicity of smokeless tobacco constituents in experimental animals

There is remarkable coherence between the carcinogenic activity in rats of tobacco-specific nitrosamines, which are constituents of smokeless tobacco, and observations in humans who use smokeless tobacco. Tobacco-specific nitrosamines – *N*′-nitrosonornicotine (NNN), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), *N*′-nitrosoanabasine (NAB), and *N*′-nitrosoanatabine (NAT) – are the most prevalent strong carcinogens in smokeless tobacco, generally found in the range of 1–10 µg per gram of product (IARC, 2007). No smokeless tobacco product analysed for these compounds has ever been reported to be free of them. Nitrosamine carcinogenesis was discovered by the pioneering work of Magee and Barnes on dimethylnitrosamine (Magee and Barnes, 1956). Subsequently, multiple studies by numerous investigators demonstrated that more than 200 nitrosamines are carcinogenic in laboratory animals, frequently inducing tumours in an organ-specific and systemic fashion, and in many cases after treatment of animals with very low doses (Preussmann and Stewart, 1984; Gray et al., 1991; Peto et al., 1991; Lijinsky, 1992). More than 30 different animal species develop cancer after treatment with various nitrosamines (Bogovski and Bogovski, 1981). Nitrosamines are genotoxic carcinogens that absolutely require metabolism to exert their carcinogenic effects (Hecht, 1998b). NNN and NNK, the most carcinogenic of the tobacco-specific nitrosamines, are typical members of the nitrosamine class of carcinogens (Hecht, 1998a). Amounts of NNN and NNK in many different types of smokeless tobacco products have been summarized based on the results of thousands of analyses; levels of NNN generally exceed those of NNK (IARC, 2007).

Multiple carcinogenicity studies of NNN have been reported (Hecht, 1998a). A recent investigation explored the carcinogenicity in rats of (*S*)-NNN, the enantiomer of NNN that is most prevalent in tobacco products, comprising 57–67%



(*S*)-NNN



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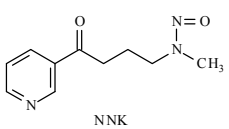
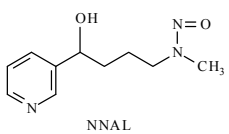
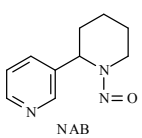


NNK



NNAL



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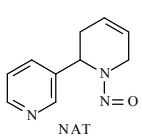

NAT

of total NNN in smokeless tobacco and cigarette tobacco (Balbo et al., 2013; Stepanov et al., 2013). (*S*)-NNN was administered in the drinking-water (15 ppm) to a group of 24 male Fischer 344 (F-344) rats. Two other groups of rats were given either (*R*)-NNN (15 ppm) or racemic NNN (30 ppm). The rats in the groups treated with (*S*)-NNN or racemic NNN began losing weight after 1 year of treatment and had died or were humanely killed by 17 months. All rats treated with (*S*)-NNN had tumours of the oral cavity. A total of 91 such tumours were observed in 20 rats that were necropsied, including tumours of the tongue, larynx, pharynx, oral mucosa, and soft palate. Some of the oral cavity tumours were large. The rats treated with (*S*)-NNN also had 122 oesophageal tumours. In contrast, (*R*)-NNN was only weakly tumorigenic. A highly significant carcinogenic response similar to that resulting from exposure to (*S*)-NNN was also observed in the rats treated with racemic NNN. The induction of tumours of the oral mucosa, tongue, larynx, and pharynx as well as oesophageal tumours in all rats treated with (*S*)-NNN or racemic NNN is remarkably consistent with the epidemiological studies of smokeless tobacco use summarized above. Although this was the first study to investigate the carcinogenicity of (*S*)-NNN, previous studies of racemic NNN administered in the drinking-water to rats uniformly produced high yields of oesophageal tumours, and oral cavity tumours were occasionally observed (Hecht, 1998a; IARC, 2007). The doses of NNN given in the earlier studies probably either were too low to observe a high incidence of oral cavity tumours in addition to oesophageal tumours, or were so high that they caused death from oesophageal tumours before oral cavity tumours could be observed.

Based on consumption of half a tin (17 g) per day of a popular smokeless tobacco product (Hecht et al., 2008a) containing about 3 µg per gram of NNN (Hecht et al., 2011) and an extraction efficiency of 60% (Hecht et al., 2008b), human exposure would be about 34 µg per day of NNN, or 20 µg per day of (*S*)-NNN; in 30 years of use, this would amount to about 220 mg (3 mg/kg body weight) of (*S*)-NNN. This compares to a dose of 150 mg (375 mg/kg body weight) of (*S*)-NNN in the drinking-water study described above. It is unclear whether a body weight correction is relevant, considering that smokeless tobacco is concentrated in the oral cavity and is frequently held at one site.

Whereas administration of NNN in the drinking-water to F-344 rats produces tumours of the oral cavity and the oesophagus, subcutaneous injection of NNN causes mainly tumours of the nasal mucosa, with malignant tumours arising predominantly in the olfactory epithelium (Hecht, 1998a). Treatment of mink with NNN by subcutaneous injection also produced malignant nasal tumours (Koppang et al., 1992; Koppang et al., 1997; IARC, 2007).

Carcinogenicity studies of NNN with Syrian golden hamsters have involved subcutaneous injection of NNN or swabbing of the cheek pouch. Tumours of the trachea and nasal cavity were observed upon subcutaneous injection; the cheek pouch was generally unresponsive (Hecht, 1998a). Treatment of various strains of mice with NNN by oral or intraperitoneal administration has resulted mainly in pulmonary adenomas (Hecht, 1998a). Thus, studies with Syrian golden hamsters and mice are generally less coherent with the epidemiology of smokeless tobacco use than are the studies in rats (IARC, 2007).

Swabbing the oral cavity and lips of rats with a mixture of NNN and NNK for 131 weeks produced 9 oral cavity tumours in 8 of 30 rats, which was statistically significant, but the result was not nearly as strong as that noted earlier, in part because the dose of racemic NNN in the swabbing study was about 40% of that described for the drinking-water study mentioned above (Hecht et al., 1986). NNK by itself did not induce oral cavity tumours when swabbed in the oral cavity of rats or hamsters (Hecht, 1998a). An interesting and unexplored observation in the swabbing study was that an extract of fine-cut moist snuff of the type used orally inhibited the oral cavity carcinogenicity of NNN and NNK.

Although the carcinogenicity studies of NNN administered orally to rats are in many respects remarkably consistent with the results of epidemiological studies of cancers of the oral cavity and the oesophagus in humans, they did not produce any pancreatic tumours. In another example of coherence, NNK and its metabolite 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) both produced significantly increased incidences of exocrine pancreatic tumours when administered in the drinking-water to male F-344 rats at doses of 1 ppm (NNK) or 5 ppm (NNAL) (Rivenson et al., 1988). It should be noted, however, that the lung was clearly the main target organ for NNK and NNAL in these studies – with significant increases in lung cancer for both agents ($P < 0.01$) – whereas the results of epidemiological studies on

smokeless tobacco use and lung cancer are, in aggregate, inconclusive at present (Boffetta et al., 2008).

Coherence: mechanistic studies of carcinogenicity of smokeless tobacco

Mechanistic studies can help bridge the gap between epidemiological investigations and experimental studies in laboratory animals. With respect to the role of tobacco-specific nitrosamines in carcinogenesis induced by smokeless tobacco products, as indicated by the animal studies described above, the first mechanistic question that arises concerns uptake of constituents. Many studies have demonstrated the presence of tobacco-specific nitrosamines in the saliva of smokeless tobacco users (IARC, 2007). In one study that quantified levels of NNK in a product before and after use, it was determined that approximately 59% of the NNK in a popular brand of smokeless tobacco was extracted during use (Hecht et al., 2008b). A second study of this type reported removal of 30% of the NNK and 23% of the NNN from an oral *snus* product during use (Caraway and Chen, 2013).

Analysis of the urine of smokeless tobacco users further demonstrates the uptake and metabolism of tobacco-specific nitrosamines. NNN, NNAL, NAT, and NAB as well as their glucuronides have all been detected in the urine of smokeless tobacco users at levels similar to or greater than those found in the urine of most smokers (Stepanov and Hecht, 2005; Hecht et al., 2007). It has been estimated that NNAL plus its glucuronides comprise 14–17% of the NNK dose in people who use a popular smokeless tobacco product, and that their uptake of NNK is about 6 µg per day (Hecht et al., 2008b). Furthermore, the level of NNAL plus its glucuronides in the urine of smokeless tobacco users is higher than that in controls and is also significantly correlated with years of use (Hecht et al., 2007).

Nitrosamines require metabolism to exert their carcinogenic effects, and the tobacco-specific nitrosamines NNN and NNK are no exception (Preussmann and Stewart, 1984; Hecht, 1998a). Many studies have conclusively demonstrated that α-hydroxylation of these compounds catalysed by cytochrome P450 enzymes leads to the formation of reactive metabolites and DNA adducts, and that these DNA adducts are crucial in the carcinogenic process. These studies have been reviewed in detail (Hecht, 1998a, 2008; IARC, 2007). As an example of the importance of DNA adducts in carcinogenesis by NNN, it is worth noting that the formation of NNN–DNA adducts in the oesophagus, oral cavity, and liver of rats treated chronically with 10 ppm of (*S*)-NNN or (*R*)-NNN in drinking-water correctly predicted cancer induction in the oral cavity and oesophagus of rats upon treatment with these enantiomers as described above (Lao et al., 2007; Zhang et al., 2009a). Thus, there is great coherence between mechanisms of NNN metabolism and DNA binding in rats and the corresponding carcinogenicity data. Less is known about mechanisms of pancreatic carcinogenesis by NNK, but DNA adducts of NNK and its metabolite NNAL have been characterized in the pancreas in rats (Zhang et al., 2009b).

In tandem with the formation of DNA adducts by NNN and NNK in experimental animals, the formation of haemoglobin (Hb) adducts occurs, because intermediates that react with DNA also react with Hb. These Hb adducts, when treated with base, release 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB) and have therefore been termed HPB-releasing Hb adducts. Their formation and persistence in rats treated chronically with either NNK or NNN has been well documented (Hecht, 1998a; IARC, 2007).

Detection of NNN–DNA and NNK–DNA adducts as well as HPB-releasing Hb adducts would be anticipated in smokeless tobacco users, but there are no published studies on NNN–DNA and NNK–DNA adducts in this group. However, several studies have reported the presence of HPB-releasing Hb adducts in humans, with the highest levels consistently seen in smokeless tobacco users (Hecht, 1998a; IARC, 2007). These studies provide evidence that NNN and NNK are metabolically activated to form HPB-releasing Hb adducts in smokeless tobacco users, although it is possible that there could be other sources of these adducts as well. Collectively, these studies demonstrate coherence between mechanisms of NNN and NNK metabolic activation in rats and in smokeless tobacco users. Thus, there is coherence in the carcinogenicity data and in the mechanistic data available for these specific compounds and the observed cancer-causing effects of smokeless tobacco in humans.

There are still some noteworthy gaps that prevent the development of a completely coherent picture of NNN metabolism in laboratory animals and humans. Multiple studies, including some of those described above, indicate that in F-344 rats, 2′-hydroxylation of NNN is important in the formation of DNA adducts and in the expression of carcinogenicity

by NNN (Hecht, 1998a; IARC, 2007). It is not known which cytochrome P450 enzyme is responsible for NNN 2′-hydroxylation in the oral cavity and oesophagus in rats, or in humans. Two human cytochrome P450 enzymes that catalyse (*S*)-NNN metabolism by 5′-hydroxylation – cytochrome P450 2A6 and 2A13 enzymes – do not catalyse the 2′-hydroxylation (Wong et al., 2005). This raises some questions about the enzymology of (*S*)-NNN metabolic activation in humans. Further, in studies of NNN metabolism in patas monkeys, the major pathway appears to be 5′-hydroxylation (Upadhyaya et al., 2002). More research is needed to determine whether these observations reflect a lack of coherence between rats and humans or simply a lack of relevant data.

As noted above, the formation of DNA adducts is critical in the carcinogenic process induced by the agents discussed here. In contrast to the plethora of information available on DNA adduct formation in laboratory animals by smokeless tobacco constituents – most commonly NNN and NNK – there is a paucity of studies on DNA adduct formation by smokeless tobacco itself, both in laboratory animals and in humans (IARC, 2007). The few studies that have been reported either used non-specific techniques or did not find consistent effects of smokeless tobacco on DNA adduct formation. Similarly, there is at present no convincing published evidence that use of smokeless tobacco produces DNA adducts in the oral cavity, oesophagus, or pancreas in humans. This represents a significant gap in a mechanistically coherent pathway to cancer upon smokeless tobacco use as observed in epidemiological studies.

Nevertheless, many studies in human users of smokeless tobacco – but fewer in laboratory animals – demonstrate genetic effects that are consistent with the consequences of DNA adduct formation. Higher frequencies of micronuclei in buccal cells of smokeless tobacco users have been reported in multiple studies (Proia et al., 2006). Mutations in important growth control genes, such as *TP53* and *RAS*, from oral cavity tumours of smokeless tobacco users have also been observed frequently and are likely to be the result of DNA damage (IARC, 2007).

Conclusions

There is considerable coherence between established target tissues for the carcinogenicity of smokeless tobacco in humans – the oral cavity, oesophagus, and pancreas – and target tissues in rats treated orally with NNN or NNK, which are constituents of all smokeless tobacco products and are present in commonly used products at concentrations higher than those of other strong carcinogens. There is also coherence between the mechanisms by which NNN and NNK induce cancer in rats, via DNA adducts and their consequent effects, and observations in humans. There is less coherence between carcinogenicity and mechanistic aspects of smokeless tobacco exposure per se in laboratory animals and humans, in part because of operational difficulties in carrying out carcinogenicity studies, and perhaps because the right questions have not been addressed with respect to mechanisms.

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