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Survey Report

The molecular basis for induction of human cancers by tobacco specific nitrosamines

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ABSTRACT

Cellular DNA damage that is misrepaired or not repaired, constitutes a necessary, although not sufficient prerequisite for induction of cancer. For carcinogenic oral snuffs with extremely high concentrations of tobacco specific nitrosamines (TSNA) the DNA adduct levels predicted from animal experiments exceed those found in "unexposed" individuals. On the other hand, and supported by extensive Swedish epidemiological data, no significant increase of TSNA-induced DNA damages can be anticipated in humans from the use of low-nitrosamine oral snuffs. The extrapolated adduct concentrations are orders of magnitude lower than those found in the corresponding human tissues, a discrepancy that is difficult to account for by species differences. Furthermore, in exposed subjects the observed *increment* in the background levels of pyridyloxobutyl(POB)–hemoglobin adducts – a relevant indicator for TSNA activation – lie in a range predicted by rodent data. When based on the same type of tissues this provides justification for extrapolating rates of TSNA induced adduct formation from animals to humans. A TSNA exposure that does not affect the background level of pro-mutagenic DNA lesions should be considered as "virtually safe". The high background concentrations of methylated and POB–DNA adducts in "unexposed" humans must be ascribed to other sources than tobacco.

1. Introduction

Cellular DNA damage that is misrepaired or not repaired constitutes a necessary, although not sufficient prerequisite for induction of cancer. Thus, IARC (2007) underlines the important mechanistic role of DNA adducts derived from tobacco specific nitrosamines (TSNA) in the induction of cancer by smokeless tobacco products. The main purpose of this review is to examine the dose–response relationships between exposure to TSNA and formation of DNA and hemoglobin (Hb) adducts in the rodent, and to compute on basis of such data the adduct concentrations to be anticipated in humans exposed to tobacco. The expected adduct levels are then compared with those that have actually been found in the corresponding human tissues, while taking available epidemiological evidence into consideration.

2. Exposure to nitrosamines from tobacco

2.1. Tobacco specific nitrosamines (TSNA)

Among the nitrosamines formed during curing and processing of tobacco, rodent data indicate that 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanone (NNK) and N′-nitrosonornicotine (NNN) have the highest carcinogenic potency, inducing tumors in lung, liver, nasal cavities, esophagus and exocrine pancreas. The TSNA N′-nitrosoanabasine is considerably less active in this respect, and for N′-nitrosoanatabine evidence of carcinogenic activity seems to be lacking (IARC, 2007a).

The levels of TSNA in smokeless tobacco may differ by orders of magnitude depending on origin and manner of processing (Table 1). Sudanese "toombak", that has been clearly associated with cancers of the oral cavity (Idris et al., 1994; Ahmed and Mahgoob, 2007), may contain up to 7870 μg/g (av. 2310 μg/g) of NNK and up to 3080 μg/g (av. 1130 μg/g) dry weight of NNN (Idris et al.,

Abbreviations: AGT, O^6-alkylguanine DNA-alkyltransferase; BaP, benzo[a]pyrene; CHO, Chinese hamster ovary; Gua, guanine; Hb, hemoglobin; HPB, 4-hydroxy-1-(3-pyridyl)-1-butanone; IARC, International Agency for Research on Cancer; NDMA, N-nitrosodimethylamine; NNAL, 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butan-1-ol; NNK, 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanone; NNN, N′-nitrosonornicotine; NOEL, no observed effect level; PAH, polycyclic aromatic hydrocarbons; POB, pyridyloxobutyl; PHB, pyridylhydroxybutyl; O6-mGua, O^6-methylguanine; O4-mTh, O^4-methylthymine; 7-mGua, 7-N-methylguanine; TN, total normal nucleotides; TSNA, tobacco-specific nitrosamines.

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Table 1
Nitrosamine content (based on dry weight) of tobacco products from various sources.

Source	NNK (μg/g)	NNN (μg/g)	Total TSNA (μg/g)
Moist oral snuff products			
Sudan, "toombak"[a]	2310 (620–7870)	1130 (500–3080)	3740 (1160–13610)
US			
1980, $n = 3$[b]	2.8 (1.3–4.7)	23 (3.5–39)	45.3 (6.6–85.4)
1987[c]	1.3 (0.1–3.1)	16.6 (5.8–64.1)	85.6 (9.6–288)
1995; Copenhagen[d]	1.89 ± 0.62 (1.45–3.20)	8.73 ± 1.44 (6.47–10.78)	17.2 ± 3,0 (13.33–22.13)
1995; Kodiak Wintergreen[d]	0.55 ± 0.15 (0.37–0.84)	6.30 ± 1.06 (4.63–8.05)	11.0 ± 2.4 (7.22–14.91)
2007; mean of 12 brands[e]	0.23 (0.08–0.36)	2.05 (1.05–3.28)	2.61 (1.33–6.19)
Canada; 2 samples, 1991[f]	3.2/5.8	50/79	209/260
Norway, 2 samples, 1983[g]	10.8/18.2	26/58	300/1100
Sweden	1.8 (0.59–3.8)	12.5 (3.5–77.1)	19.8 (5.5–106)
1980, $n = 13$[b]	3.2 (1.0–4.8)	7.6 (5.6–10.6)	14.6 (11.0–20)
1983, $n = 32$[h]	0.32	0.96	1.90
2002 Swedish Match, $n = 7$[i]	(0.26–0.38)	(0.84–1.12)	(0.3–6.0)
Dry oral snuff products			
3 US brands[c]	9.4 (2–14)	33.6 (9–55)	72.9 (31–111)
Cigarette tobacco			
3 US modern brands[i]	1.57 ± 0.18 (1.41–1.76)	3.32 ± 0.88 (2.59–4.30)	6.10 (3.3–8.9)
Moldovian cigarettes, 18 brands[i]	0.19 ± 0.09 (0.10–0.48)	0.58 ± 0.55 (0.09–2.09)	0.94 ± 0.66 (0.25–3.08)
French Gauloises[j]			8.7

[a] Idris et al. (1991).
[b] Hoffmann and Adams (1981).
[c] Adams et al. (1987)
[d] Hoffmann et al. (1995).
[e] Stepanov et al. (2008a).
[f] Brunnemann and Hoffmann (1991).
[g] Österdahl and Slorach (1984).
[h] Österdahl et al. (2004)
[i] Stepanov et al. (2002).
[j] Hewett (1987).

1991). These extreme values correspond to a daily total absorbed dose from 20 g moist oral snuff (50% water; 60% absorption, 70 kg BW) of about 53–674 μg/kg/day of NNK and 43–264 μg/kg/day of NNN. In South East Asia locally grown tobacco that sometimes is heavily roasted (*gutka, mishri*), and/or mixed with areca nuts and betel leaves (*betel, zarda*, *dokta*, *mawa*) is used in many products for oral use. In addition to high levels of TSNA (Stepanov et al., 2005), they may contain arecoline and associated nitrosamines as well as other carcinogenic impurities, notably significant quantities of polycyclic aromatic hydrocarbons (PAHs) (IARC, 1985, 2007a), a fact that underlines the highly variable composition and properties of smokeless tobacco from different sources (Table 1).

In comparison with snuff from Sweden, two samples of Norwegian snuff from 1983 (Österdahl and Slorach, 1984) and two from Canada from 1991 (Brunnemann and Hoffmann, 1991) exhibited a relatively high content of NNK (Norwegian 5.4/7.8; Canadian 3.2/5.8 μg/g) and NNN (Norwegian 26/58; Canadian 50/79 μg/g). The levels of nitrosamines in these smokeless tobacco products should be compared with today's Swedish snuff ("snus") with a total TSNA content of about 2 μg/g dry weight (Österdahl et al., 2004). However, analysis performed by Hoffmann and Adams (1981), as well as the reduction of the content of volatile nitrosamines upon introduction of a new production process based on heat sterilization of tobacco (Österdahl and Slorach, 1983, 1984) indicate, that Swedish snuff marketed prior to 1983 had higher contents of NNK and NNN than those found in the first analyses to be performed by the Swedish National Food Administration (Österdahl and Slorach, 1984). By 1983 the average levels (dry weight) were 3.2 μg/g of NNK and 7.6 μg/g of NNN (daily intake: 0.27 NNK and 0.65 μg/kg NNN). Subsequently, further reductions occurred. According to recent analyses performed by the Swedish National Food Administration, Swedish snuff produced by Swedish Match (accounts for 99% of the Swedish market) today contains on average 0.32 μg/g of NNK and 0.96 μg/g of NNN, giving daily total uptakes for consumption of 20 g snuff of 0.027 for NNK and 0.082 μg/kg/day for NNN, respectively (Österdahl et al., 2004).

But for a few exceptions, the levels of TSNA in US brands were formerly consistently higher than those found in Swedish snuff. However, during the last decade there has been a considerable reduction of the nitrosamine contents of US snuffs, where 12 different brands purchased in 2007 had a mean total TSNA content of 2.61 μg/g (Stepanov et al., 2008a).

Similar to smokeless tobacco, the variations in TSNA content of cigarette tobacco are appreciable. Greek and Turkish types of tobacco are generally characterized by low TSNA contents (Stepanov et al., 2002; D'Andres et al., 2003). French Gauloises had 8.6 μg/g of TSNA, whereas in US and German brands the TSNA concentrations were in the range 1.6–5.5 μg/g (Hewett, 1987). For estimation of actual intakes, measurements of average yields in mainstream smoke from one cigarette (as per 2 mg nicotine) conducted at MIT are probably representative (Harris, 2004), and shall be used in this context. This source indicates an uptake of about 0.25 μg of NNN and 0.17 μg of NNK per cigarette assuming 100% absorption in the lung. Smoking 20 cigarettes per day will then give a

dose of 0.07 μg/kg/day of NNN and 0.05 μg/kg/day of NNK for a person weighing 70 kg. In a study in 182 users of US smokeless tobacco of unspecified quality and 420 smokers the exposures to NNK were somewhat higher in users of smokeless tobacco (Hecht et al., 2007a).

The daily intakes presented above provide approximate benchmark exposures when estimating levels of pro-mutagenic DNA adducts to be expected in humans when based on rodent data.

2.2. Other nitrosamines and polycyclic aromatic hydrocarbons (PAH)

In comparison with NNK and NNN the volatile nitrosamines nitrosodimethylamine (NDMA), N-nitrosopiperidine, and N-nitroso-pyrrolidine are formed in quantities that are orders of magnitude lower than the TSNA. Thus, the concentration of NDMA in Swedish oral snuff and chewing tobacco tobaccos were in the ng/g range (Österdahl and Slorach, 1984), and Tricker et al. (1991) report a value of 2.0 ng per non-filter cigarette.

Similarly to some foods, small quantities of the weakly carcinogenic non-tobacco specific nitrosoamino acids N-nitrososarcosine, 3-(methylnitrosamino)-propionic, and the moderately active 4-(methylnitrosamino)-butyric acid are also formed in snuff upon processing and storage (Österdahl and Slorach, 1983; Tricker et al., 1991). However, the contribution of these nitrosamines to total cancer risk seems to be negligible.

In contemporary Swedish "snus" that is based on a tobacco that is neither fermented nor fire cured, the PAH content is very low (Ahlbom et al., 1997; Rutqvist, 2010). However, in 17 samples of US smokeless tobacco the sum of 23 detected PAH averaged 11.6 μg/g dry weight (Stepanov et al., 2010). Previously, concentrations up to about 60 ng/g of the potent carcinogen benzo[a]pyrene (BaP) have been reported for US smokeless tobacco (Hoffmann et al., 1986), a value that is in good agreement with the aforementioned recent results. As a comparison, the Swedish State Food Administration found BaP levels up to 212 ng/g in Frankfurter sausages grilled over open fire (Larsson et al., 1983), results that we have been able to verify for grilled pork meat (EU DG Research, 2007).

3. Metabolic transformations of TSNA

Based on the excretion of nicotine and its metabolites (Andersson et al., 1994) as well as by estimating the area under the venous plasma concentration–time curve (AUC) (Lunell and Lunell, 2005), a maximal nicotine absorption of about 60% has been demonstrated for several brands of Swedish snuff. In this context it seems reasonable to utilize this value also for TSNA in smokeless tobacco. The complex metabolic patterns of NNK and NNN have been extensively reviewed elsewhere (Hecht, 1996, 1998, 2008; Richter et al., 2009a), and only the most salient features will be mentioned here.

The metabolic transformation steps mediated by CYP450 enzymes (Jalas et al., 2005) critical for the generation of DNA and hemoglobin adducts consist in the production from NNK of the directly methylating agent, methanediazohydroxide, that upon reaction with DNA give rise mainly to 7-N-methylguanine (7-mGua) and O^6-methylguanine (O6-mGua) as well as small amounts of O^4-methylthymine (O4-mTh). Reduction of the carbonyl group of NNK leads to the formation of 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butan-1-ol (NNAL) which may form a glucuronide, or undergo methylene hydroxylation like for NNK. The excretion of NNAL and its glucuronide represents a useful index of exposure in users of tobacco (Carmella et al., 1993; Meger et al., 1996; Hecht, 2002).

A second pathway leads to the formation of 4-(3-pyridyl)-4-oxobutane-1-diazohydroxide that introduces pyridyloxobutyl (POB) adducts in DNA and proteins like hemoglobin (Carmella and Hecht, 1987; Carmella et al., 1990; Hecht et al., 1991; Falter et al., 1994). Mild acid or alkaline hydrolysis of these adducts releases 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB), which can be analyzed by GC–MS. Oxidative metabolism of NNN generates the same reactive diazohydroxide as is obtained upon α-hydroxylation of the terminal methyl group of NNK, thereby introducing POB in proteins and DNA. NNN gives a complex pattern of metabolites in urine (Hecht et al., 1981; Upadhyaya et al., 2002; Stepanov and Hecht, 2005). It should also be noted, that N′-nitrosonornicotine has been found in the urine of users of nicotine replacement products, indicating endogenous formation of NNN from nicotine (Stepanov et al., 2009). Fig. 1 gives a schematic presentation of the activation pathways of TSNA, where a possible role of the secondary tobacco alkaloid myosmine is also included (discussed below).

4. Carcinogenicity of smokeless tobacco

The carcinogenic action of Sudanese oral snuff with extremely high levels of TSNA was mentioned above (Idris et al., 1991, 1994; Ahmed and Mahgoob, 2007). Further, there is sufficient evidence from South East Asia for an association between cancers in the head–neck region and several forms of oral tobacco (IARC, 1985, 2007a). A case–control study from 1980 (Winn et al., 1981), that seems to be one of the few adequately performed larger studies in the US, demonstrated a significant elevated risk for cancers of the oropharynx in white women from North Carolina who had used a locally produced dry snuff of unknown purity for a long time, and where a major part of the exposure had occurred before World War II. Later studies conducted in US based on a limited material gave conflicting results (Rodu and Jansson, 2004; Lee and Hamling, 2009).

Moist oral snuff ("snus") has been widely used in Sweden for almost 200 years, and the high consumption of snuff during recent years has been accompanied by a drastic reduction in smoking that is the lowest among industrialized countries. The prevalence of smoking among all men in northern Sweden is now about 9% (C.I. 7.0–11%) and only 3% (CI 0.1–5.4%) among men age 25–34 years; the prevalence of exclusive snus use is 27% (C.I. 24–30%) and 34% (C.I. 27–42%), respectively (Stegmayr et al., 2005).

The high prevalence of snuff use in the Swedish population, as well as the existence of a comprehensive cancer registry, offers a unique possibility to study the impact of TSNA on health. In several large case–control as well as cohort investigations that have been carried out in this country no significant increase in cancers in the head–neck region or lung was detected (Lewin et al., 1998; Schildt et al., 1998; Rosenquist et al., 2005; Luo et al., 2007). Based on a mere 11 cases an increase in the combined incidences of oral and pharyngeal cancer was found in a Swedish cohort of users of snuff (Roosaar et al., 2008), and Zendehdel et al. (2008) reported a statistically significant excess risk for esophagal and stomach cancers in a Swedish retrospective cohort study. Several other studies failed to confirm the latter finding (Hansson et al., 1994; Lagergren et al., 2000; Bofetta et al., 2005), and in view of possible smoker misclassification and failure to adjust for alcohol abuse, no valid conclusions can be based on the Zendehdel et al. (2008) study. Two studies reported a statistically significant association between snuff use and risk for pancreatic cancer (Bofetta et al., 2005; Luo et al., 2007). However, confounding by alcohol abuse and diabetes, two important risk factors, were not controlled for. With the participation of Boffetta, a pooled analysis of smokeless tobacco use and risk of pancreatic cancer using data from 11 case–control studies (6056 cases and 11,338 controls) within the International Pancreatic Cancer Case–Control Consortium failed to reveal any significant association for smokeless tobacco use. As reason for an outcome that differed from the above mentioned Scandinavian studies, a lack of appropriate control of confounding in the latter was given (Bertuccio et al., 2011). A recently

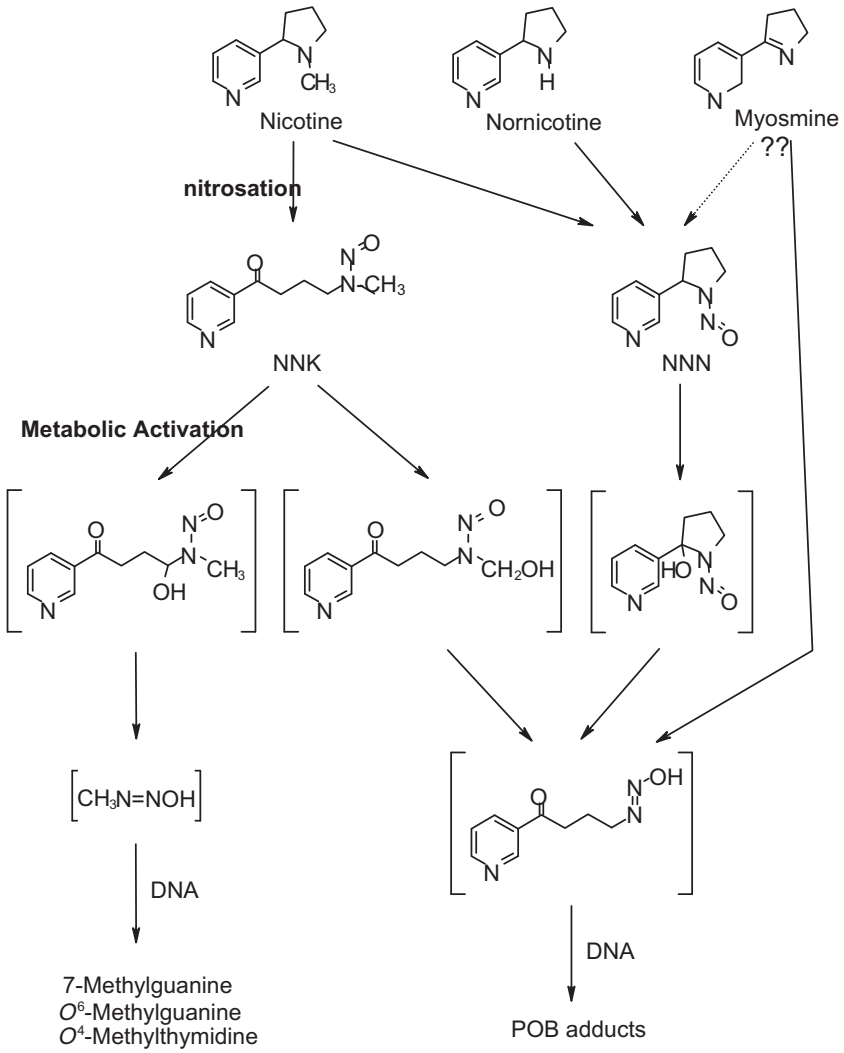

Fig. 1. Schematic presentation of the activation pathways of TSNA.

conducted comprehensive meta-analyses failed to indicate any association between cancers at different sites and the use of Swedish-type moist snuff (Lee, 2011).

Data from bioassays with smokeless tobacco *per se* have been reviewed in detail by Grasso and Mann (1998) as well as by IARC (2007a); the outcome of the majority of these studies have been negative.

5. Type and biological significance of DNA adducts induced by TSNA

NNK and NNN induce three types of crucial DNA damages: nucleotide methylations, pyridyloxobutylations (POB) as well as pyridylhydroxybutylations. In addition, DNA phosphate POB adducts have been identified (Haglund et al., 2002). 7-N-methylguanine (7-mGua) is the predominant adduct found in target tissues induced by NNK, followed by O^6-methylguanine (O6-mGua), whereas very low levels of O^4-methylthymine (O4-mTh) are present (Belinsky et al., 1986). Depending on the presence of activating enzymes, adduct formation from NNK show great variations between rodent tissues (Deilhaug et al., 1985; Belinsky et al., 1987a, 1988; Jansen et al., 1996).

The capacity of various DNA adducts to induce mutations and chromosomal aberrations vary extensively. However, O6-mGua is a highly pro-mutagenic adduct causing G:C to A:T transitions (Jansen et al., 1996; Margison et al., 2002) induced, e.g. by NNK in the Ki-ras gene of A/J mouse lung tumors (Belinsky et al., 1989; Ronai et al., 1993). O6-mGua appears to play a major role in lung tumorigenesis induced by TSNA in rodents (Belinsky et al., 1987b, 1990; Peterson and Hecht, 1991; Upadhyaya et al., 2009), as well as in several types of human cancers (Margison et al., 2002).

Although the concentrations of O4-mTh induced by methylating agents in the rat are more than one order of magnitude below those for O6-mGua (Den Engelse et al., 1986; Belinsky et al., 1986), they may contribute to a limited extent to the over all cancer risk from TSNA because of their mutagenic potential. On the other hand, after NNK treatment O6mGua persisted, while O4-mTh was removed rapidly in the lung, suggesting operation of different repair pathways (Belinsky et al., 1986).

Phosphate alkylations do not appear to contribute significantly to the overall genotoxicity of TSNA (Törnqvist, personal communication).

Distortion of the DNA helix by 7-mGua is insignificant, and the DNA polymerases will not distinguish its presence from the normal nucleoside upon replication. 7-mGua is rapidly removed by base excision repair or by spontaneous depurination giving rise to apurinic sites that are prone to undergo rapid and error-free repair (Plosky et al., 2002). Thus, 7-mGua, which has a low mutagenic potency, seems to be of secondary importance with respect to cancer induction by NNK or NNN (Peterson and Hecht, 1991). This

assumption is further strengthened by the observation that, in contrast to O6-mGua, there was no correlation between persistence of 7-mGua adduct levels from NNK and incidence of liver tumors in the rodent (Liu et al., 1992).

Some of the POB–DNA adducts have recently been identified, and include 7-POB guanine, O^2-POB thymine, O^6-POB guanine as well as O^2-POB cytosine, where 7-POB guanine and O^2-POB thymine represent the major adducts (Lao et al., 2007; Zhang et al., 2009). The mutagenicity of the POB adducts apparently shows considerable variation. Like 7-mGua, 7-POB guanine has been reported to undergo rapid spontaneous depurination generating apurinic sites (Wang et al., 2003). However, it seems that O^2-POB thymine is a likely candidate (Li et al., 2009) involved in the induction of cancers mainly found in the respiratory mucosa and esophagus observed at high NNN doses in the rat (Griciute et al., 1986). Thus, in a model system where POB adducts were induced by acetoxymethylnitrosamino pyridyl butanone in repair proficient and repair deficient Chinese hamster ovary cells, analysis of the mutation spectra in the *hprt* locus identified AT base pair point mutations to predominate, whereas G:C to A:T transitions were less frequent. In nucleotide excision repair deficient cells repair of O^2-POB thymine adducts was reduced, whereas deficiency of AGT had no impact (Li et al., 2009). In the p53 gene in oral squamous-cell carcinomas from 14 users of Sudanese toombak, there were 15 transversions, 9 transitions, 3 insertions and one deletion (Ibrahim et al., 1999).

Kinetic data indicate a dose threshold for POB–DNA adduct formation in the lung (Murphy et al., 1990), and the fact that NNN by high dose oral administration has an overall carcinogenic potency (0.029 per mg/kg and day) that is about one third of that for NNK (0.086 per mg/kg and day) (Nilsson, 1998), indicate that in comparison with O6-mGua the POB adducts are less efficient in cancer initiation.

6. Kinetics of adduct formation in the rodent

Investigation of the complex kinetics of DNA adduct formation induced by NNK and NNN has revealed marked differences between tissues and cell types. Further, exposure to NNK by the oral route may in some cases result in a tissue adduct distribution, as well as critical site for cancer induction, that is different from that from s.c. or i.p. injection. Thus, in contrast to injection, pancreatic tumors can be induced by administering NNK or its metabolite NNAL by the oral route (Rivenson et al., 1988). Whereas the levels of O^6- and 7-mGua adducts formed from NDMA (1–10 μg/kg) in rat kidney were 4–10 times lower upon oral administration than by the i.p. route, no such differences were on the other hand found for liver (Pegg and Hui, 1978).

For the purpose of comparative quantitative risk assessment, data on induction of DNA and hemoglobin adducts in experimental animals become especially meaningful in this context when used (i) to estimate what adduct levels to be expected in users of tobacco, (ii) to compare the expected adduct levels with those that have actually been measured in humans in corresponding tissues, and (iii) to relate adduct levels to tumor incidence in different tissues.

6.1. O^6–Methylguanine

O6-mGua is repaired by a "suicidal" O^6-alkylguanine DNA-alkyltransferase (AGT) that is consumed during this process, and the rates of repair vary widely between tissues (Deilhaug et al., 1985; Belinsky et al., 1988; Souliotis et al., 2004).

6.1.1. Liver

After exposure to NNK by s.c. injections 3 times per week for 4 weeks, the existence of a dose threshold for O6-mGua in liver is apparent, that most probably is due to rapid induction of the AGT in this tissue. No increase in the concentration of this adduct could be detected one day after single s.c. injections of NNK at 30–300 μg/kg, nor after s.c. injections of 1000 μg/kg 3 times/week (426 μg/kg/day) during 4 weeks. As the dose was increased to 4260 μg/kg/day, the adduct levels increased and reached a maximum alkylation efficacy of about 0.1 O6-mGua/10^9 total normal nucleotides (TN) per μg NNK/kg/day). At these high doses necrotic changes and subsequent development of hepatic neoplasia appeared after 20 weeks' treatment. 426 μg/kg/day of NNK appears to represent the NOEL for the formation of O6-mGua in liver after repeated exposure under these conditions (Belinsky et al., 1990). However, after chronic oral administration an uptake of about 700 μg/kg/day resulted in an adduct level of 0.3 O6-mGua/10^9 TN (2550 fmol/mg DNA), well above the detection limit, indicating that a lower NOEL may exist after long-term administration with drinking water (Upadhyaya et al., 2009).

6.1.2. Nasal mucosa

When rats were treated during 4 weeks by s.c. injections, 3 times per week, with doses corresponding to 13–21,400 μg/kg/day, adducts could not be detected, in either the olfactory, nor in the respiratory nasal mucosa at the lowest dose. The adduct concentrations increased rapidly in the range 43–10,000 μg/kg/day, followed by a decline in alkylation efficiency at the highest dose where a considerable degree of necrosis was found. The adduct levels in the respiratory mucosa was significantly higher than in the olfactory mucosa. After 20 weeks treatment a significant increase in malignant tumors was only detected at 10 mg/kg and above. For the rat nasal olfactory epithelium, some necrotic changes were detected at 1 mg/kg, and further necrotic changes became increasingly severe at doses above 10 mg/kg. The respiratory epithelium was considerably less sensitive. The authors therefore concluded, that cell proliferation secondary to toxicity is required for tumor induction by NNK in the rodent nasal passages (Belinsky et al., 1987a, 1990).

6.1.3. Lung

In contrast to liver and nasal mucosa, repeated administration of NNK by s.c. injection causes a progressive accumulation of O6-mGua in rat lung down to the lowest administered dose (Belinsky et al., 1987b, 1990). For this adduct there was also a good correlation between degree of alkylation in bronchiolar Clara cells after administration of NNK and the incidence of lung tumors in the mouse (Peterson and Hecht, 1991) as well as in the rat (Belinsky et al., 1990).

In Clara cells a small elevation in the levels of this O6-mGua could be detected from s.c. administration (3 times/week) of NNK to rats of a dose corresponding to 43 μg/kg/day for 4 weeks. After 20 weeks' treatment, a steep increase in the incidence of lung tumors was detected above this dose level. The alkylation efficiency appears to be linear in the dose range 43–430 μg/kg/day, where Clara cells exhibits a very high alkylation efficacy (40 O6-mGua/10^9 TN per μg NNK/kg/day). As the dose is elevated above this level, alkylation efficacy decreases sharply. For Type II cells and whole lung the corresponding values were much lower (0.7 and 1.3 O6-mGua/10^9 TN per μg NNK/kg/day, respectively) (Belinsky et al., 1990, 1991). The difference in adduct persistence between pulmonary bronchiolar Clara cells and alveolar Type II cells also seems to reflect differences in AGT activity (Deilhaug et al., 1985; Belinsky et al., 1988). On the other hand, no such marked differences in adduct levels between type II and Clara cells was found in A/J mice (Belinsky et al., 1991). When rats were given 10 ppm NNK in drinking water (~0.7 mg/kg/day) for 1–20 weeks (Upadhyaya et al., 2009), a maximal level of O6-mGua in whole lung DNA reached 2550 fmol/mg DNA (0.8 O6-mGua/10^9 TN per

μg NNK/kg/day) after 5 weeks, which is in good agreement with the data from s.c. administration considering the analytical precision for these measurements at low doses.

6.2. 7-Methylguanine

Although of secondary importance for predicting carcinogenicity, but due to its much higher rate of formation, the levels of 7-mGua can be utilized as an index of exposure (Belinsky et al., 1986; Murphy et al., 1990; Zhao et al., 1999). When rats were given 4 daily i.p. injections of tritium labeled NNK in the dose range 75–5000 μg per kg/day, there was a linear increase of the levels of 7-mGua in liver, giving an efficacy of adduct formation of approximately 0.05 pmol 7-mGua/μmol Gua per μg NNK per kg bw and day (12.5 7-mGua/10^9 TN per μg/kg/day). In lung the dose–response relationship was approximately linear in the lower dose range up to a 600 μg per kg/day, above which there was a sharp decrease in alkylation efficacy (Murphy et al., 1990). Compared with the adduct levels in liver, the concentrations in blood white cells from rats given a single dose of 150 mg NNK per kg were about two orders of magnitude lower. Interestingly, there was no difference between per os as compared to i.p. administration (Bianchini and Wild, 1994). The alkylation efficacy as found in leukocytes at this single high dose was 0.01 adducts per 10^9 TN per μg/kg/day. The efficacy of adduct formation could be higher at lower exposures, and for this reason an alkylation efficacy of 1.3 adducts/10^9 TN per μg/kg/day is used here, which is one tenth that found for lung by Murphy et al. (1990). Although most probably an overestimation, it is more in line with the relation between the corresponding levels for 7-mGua detected in humans.

6.3. POB–DNA adducts

The interpretation of POB–DNA adduct data is complicated by the fact that more than one type of adduct is generated that could have different toxic and genotoxic modes of action (Li et al., 2009). 7-POB guanine and O^2-POB thymine were found to be the major adducts (Zhang et al., 2009). Dose–response relationships in the low-dose region that can be correlated to induction of tumors by NNN are unfortunately not available. After long-term exposure to NNN via drinking water, POB adduct levels in the nasal respiratory mucosa in rats were much higher than in the nasal olfactory or oral mucosa. When investigating HPB released from liver and lung DNA in rats given daily i.p. injections of labeled NNK during 4 days, no increase in the HPB-releasing adducts could be detected in liver and lung at a dose of 3 μg/kg/day, but the dose–response curve was roughly linear in the range 15–600 μg/kg/day, and characterized by an efficacy of adduct formation of approximately 1 pmol POB/μmol Gua per mg NNK/kg/day (0.25 POB/10^9 TN per μg/kg/day) (Murphy et al., 1990).

The (S) form of NNN is the major enantiomer (75%) in tobacco (Carmella et al., 2000). When rats were given 10 ppm (S) NNN in drinking water for 20 weeks (~0.7 mg/kg/day), the levels of total POB–DNA adducts were highest in the esophagus followed by lung and liver (Lao et al., 2007). After the same exposure regime with the (R) form, the highest adduct concentration was found in lung, followed by esophagus and liver. For lung the maximum values were 380 for the (S) enantiomer, and 1370 fmol POB/mg DNA for the (R) form after 16 weeks treatment, i.e. 0.16 adducts/10^9 TN per μg/kg/day and 0.59 adducts/10^9TN per μg/kg/day, respectively. Assuming linear extrapolation down to zero dose total POB activation in lung is estimated to be about 0.27 POB/10^9 TN per μg/kg/day for crude NNN.

Experimental studies in rodents do suggest, that POB–DNA adducts derived from NNN are involved in causing cancer of the nasal epithelia, esophagus and pancreas in rodents at high TSNA exposures (Hoffmann et al., 1984; Griciute et al., 1986; Trushin et al., 1994). At least some of these adducts are most probably rapidly lost by depurination followed by efficient repair (Wang et al., 2003), and a dose threshold for accumulation of adducts seems to exist in the rat nasal epithelia where saturation of repair combined with cytotoxicity may be determining factors for the development of neoplasia (Belinsky et al., 1987; 1990).

6.4. Hemoglobin (Hb) adducts

As mentioned above, pyridyloxobutylation also produces Hb adducts that are released as hydroxypyridyl butanone (HPB) upon hydrolysis. Plotting the data for dose vs. total globin bound adducts from tritiated NNK administered to rats by 4 daily i.p. injections in the dose range 4–10,000 μg/kg/day gives a linear relationship with an efficacy of total adduct formation of about 0.72 fmol POB/mg globin per μg NNK/kg (Murphy et al., 1990). However, according to Murphy et al. (1990) only between 17% and 40% of the totally bound tritium was released as HPB, whereas Carmella and Hecht (1987) reported that 10–15% of the bound tritium in the globin of rats treated labeled NNK was released upon hydrolysis. Further, the data of Murphy et al. (1990) were derived from administration during a period of only 4 days, whereas recorded human POB Hb data reflect accumulation over the majority of the life time of the erythrocyte, which in the rat (depending on strain) lies in the range 60–70 days (Derelanko, 1987). After chronic administration to rats, a steady state seems to be achieved after 40 days, where the adduct levels were increased by approximately a factor of 6 as compared to 4 days' treatment (Carmella and Hecht, 1987). In addition, compensation should be made for the longer life time of human erythrocytes, giving an estimated 12 times higher total expected accumulation as compared the POB-Hb induction rate during 4 days (= 1.7 fmol POB/mg globin/μg NNK/kg/day) assuming that 20% of the total radioactivity is due to POB adducts. Upon administration of labeled compounds the initial total binding to rat Hb for NNN was about half of that for NNK (formation rate = 0.9 fmol POB/mg globin per μg NNN/kg/day) (Carmella and Hecht, 1987).

7. Adduct levels found in humans

7.1. O^6-Methylguanine and O^4-methylthymine

Due to lack of sensitivity of most analytical procedures few studies on the levels of O6-mGua have been conducted in humans. Using a relatively insensitive immunoassay Hecht et al. (1987) were unable to detect any increase in O6-mGua in exfoliated cells from the oral mucosa in users of snuff. In lung tissue samples from 17 autopsied subjects with known occupation and smoking history, Wilson et al. (1989) found O6-mGua levels ranging from 25 to 380 adducts per 10^9 TN employing HPLC-linked synchronous fluorescent spectrophotometry and an ultrasensitive enzyme radioimmunoassay. There were no significant differences between smokers and non-smokers. Using the ^{32}P-postlabelling method the reported concentrations of O6-mGua in liver from autopsy cases were found to be in the range 28–168 adducts per 10^9 TN. In peripheral leukocytes from healthy volunteers the median levels were 1.8–12 adducts/10^9 TN (Kang et al., 1995). In colorectal tissues O6-mGua was detected in 27 of 62 samples (detection limit 2.5 adducts/10^9 TN) where the concentrations ranged from <2.5 to 235 for normal and <2.5 to 38 adducts/10^9 TN for tumor samples (Povey et al., 2000b), Utilizing a sensitive competitive repair assay for O6-mGua, this adduct was found in 83–86% in samples of maternal and cord blood leukocyte DNA from healthy smoking and non-smoking women, and the mean values were 11–12 adducts/10^9 TN. Smoking status had no effect on the detected adduct levels (Georgiadis et al., 2000).

Similar to rats treated with NNK, the concentrations of O4-mTh in human tissues appears to be low. Thus, in human liver the mean value of the ratio between O6-mGua and O4-mTh was about 6 adducts/10^9 TN (Kang et al., 1995).

7.2. 7-Methylguanine

Using a procedure where the adducts are not isolated before ^{32}P-postlabelling, and consequently cannot distinguish between the co-migrating 7-methyl and 2-hydroxyethyl guanine adducts, an elevation of "*7-mGua*" adduct levels in a small number of tissues from smokers was reported as compared to non-smokers (Mustonen and Hemminki, 1992; Mustonen et al., 1993; Szyfter et al., 1996). However, using an improved postlabelling technique (Zhao et al., 1999) by which 7-mGua could be distinguished from the contribution from 7-hydroxyethylguanine, the differences between smokers and non-smokers from lung cancer subjects were considerably reduced. Kumar and Hemminki (1996) found that the level of 7-(2-hydroxyethyl)-guanine was twice that of 7-methylguanine in total white blood cells, whereas in isolated lymphocytes its concentration was at least fourfold higher. Thus, the level of 7-Gua in white blood cells was 320 in smokers ($n = 11$) vs. 250 adducts/10^9 TN in non-smokers ($n = 8$). For tissues from lung cancer patients the corresponding values were 650 for smokers ($n = 7$) and 400 adducts/10^9 TN for non-smokers ($n = 2$). Obviously, in the latter case the small sample size precludes any conclusions to be made. Further, using an assay where 7-mGua was isolated prior to postlabelling, no significant impact of smoking could be detected in 8 separate lung segments from 10 autopsy donors. The mean adduct level was 63 ± 58 7-mGua adducts/10^9 TN (Blömeke et al., 1996). By means of HPLC-postlabelling 7-mGua was determined in DNA from bronchial lavage cells in a cohort of 38 individuals, and although there were great individual variations, the adduct concentrations were found to be significantly higher in smokers than in never smokers, 250 ± 510 adducts/10^9 TN versus 15 ± 13 adducts/10^9 TN ($P = 0.02$; non-parametric statistics). Adduct levels were highest (1860 ± 330 adducts/10^9 TN) in individuals with the genotypes (*GSTM1 null*/*GSTT1 null* or *GSTP1 ile*/*ile*) (Lewis et al., 2004).

7.3. POB–DNA adducts

Although POB–Hb adducts have been used as a measure of exposure, for cancer initiation only the HPB-releasing DNA adducts are relevant. However, most of the previously utilized analytical procedures lacked the required sensitivity for determination of specific POB–DNA adducts in human tissues. Employing an ultrasensitive method for the determination of HPB-releasing DNA adducts by gas chromatography-high resolution mass spectrometry, the levels of HPB-releasing DNA adducts were found to be significantly higher in lung tissue from 21 cancer patients who were smokers (121 ± 77 adducts/10^9 TN) as compared to 11 non-smokers (18 ± 17 adducts/10^9 TN). Thirty of the 32 recruited patients had a diagnosis of lung cancer. However, there was a poor correlation between POB–DNA adducts and the index of exposure to TSNA as represented by HPB-releasing Hb adducts (Hölzle et al., 2007).

On the other hand, among sudden death victims there were no statistically significant differences between mean POB–DNA adduct levels in non-smokers and smokers with respect to lung (18.4 ± 19.9, $n = 56$ vs. 27.2 ± 44.4, $n = 32$), esophagus (39.3 ± 39, $n = 53$ vs. 41.4 ± 62.4, $n = 29$) and cardia (35.1 ± 33, $n = 18$ vs. 28.1 ± 27.6, $n = 12$). Further, in females the trend was consistently the reverse with higher adduct levels in lung tissue from non-smokers than in smokers; for lung (24.1 ± 27.5, $n = 14$ vs. 13.8 ± 21.7, $n = 8$), esophagus (36.6 ± 33.6, $n = 14$ vs. 42 ± 52.8 m, $n = 7$) and cardia (34.5 ± 41.7, $n = 6$ vs. 19.5/94.5). However, for cardia only two specimens were available. The considerably higher levels found in esophagus and cardia were unrelated to smoking status, and whereas there was a highly significant correlation between adducts in esophagus and cardia, no correlation was found between lung and the last mentioned sites (Schlöbe et al., 2008).

NNK and NNAL have been detected in pancreatic juice from smokers (Prokopczyk et al., 2002), but it has not been possible to detect HPB-releasing DNA adducts in pancreatic tissue from smokers (Prokopczyk et al., 2005). However, the assay used may not have been sufficiently sensitive.

Recent findings indicate an effective activation of TSNA in human buccal cells from users of low nitrosamine Swedish "snus" (Richter et al., 2009b). POB–DNA adducts were found in all oral mucosa samples, and the levels in users of snuff (5280 ± 372 adducts/10^9 TN; $n = 33$) were almost twice as high as those found in smokers (3222 ± 120 adducts/10^9 TN; $n = 90$), while non-smokers ($n = 45$) exhibited adduct levels one fifth of those in smokers (600 ± 102 adducts/10^9 TN).

7.4. POB–hemoglobin adducts

POB–hemoglobin adducts are not repaired, and the levels represent the integrated cumulative exposure during the 120 days' life-time of the human erythrocyte. Although markedly increased levels of POB–Hb adducts have been reported for smokers as compared to non-smokers in several studies, these were only 2–3 times higher in smokers. Thus, POB Hb levels increased on the average from 0.029 in non-smokers to 0.080 fmol HPB/mg globin in smokers with an estimated uptake of 0.07 μg NNN and NNK per kg and day, while in users of oral snuff of unknown purity the mean Hb adduct level was found to be 0.52 fmol POB/mg globin (Carmella et al., 1990). Significantly elevated levels of POB–hemoglobin adducts were also found in users of nasal dry snuff (0.2 fmol/mg Hb) (Falter et al., 1994).

8. Comparison of rodent and human adduct data

In Tables 2A and 2B the contents of NNK and NNN in selected tobacco products are summarized together with corresponding estimated uptakes. The TSNA data (μg/kg dry weight) based on intakes per 70 kg bw from different kinds of tobacco assuming a daily consumption of 20 g moist snuff (50% water) and 60% absorption. Intakes from smoking 20 cigarettes per day were based on TSNA concentrations in mainstream smoke according to Harris (2004).

Table 3 provides the actual adduct concentrations (adducts/10^9 total normal nucleotides, TN) found in human tissues for O6-mGua (3A), 7-meGua (3B), POB–DNA (3C) as well as POB–Hb adducts (3D). These levels are compared with those expected on basis of data from the corresponding tissues in rats exposed to NNK and NNN. The calculated uptakes (as explained in Section 1) were multiplied by the specific alkylation efficacy found for the corresponding rodent tissue (expressed as adducts/10^9 TN per μg TSNA intake/kg and day, or as fmol HPB/mg globin for Hb adducts) (Section 6).

These comparisons indicate, that for tissues where information from humans are available, the rodent data do indeed predict Sudanese Toombak to increase the levels in humans of all of the listed biomarkers, thereby posing a risk for development of neoplasia.

On the other hand, the extrapolated DNA concentrations for O6-mGua, 7-meGua and POB–DNA adducts from exposure to Swedish snuff, as well as to representative contemporary US cigarettes, are more than 2 orders of magnitude lower than those actually observed in humans.

When conducting analysis of hemoglobin from rodents as well as humans, basically the same analytical methodology has been used. In the studies of dose response by Murphy et al. (1990) using radiolabeled NNK and by Carmella and Hecht (1987) utilizing

Table 2A
Contents and estimated uptakes of NNK in humans from use of selected tobacco products.

Type of tobacco	NNK content (μg/g)	Estimated total human uptake (μg/kg bw/day)	Reference
Sudanese "Toombak"	620–7870	53–674	Idris et al. (1991)
Swedish snuff 1980	1.8	0.16	Hoffmann and Adams (1981)
Swedish snuff 1983	3.2	0.28	Österdahl and Slorach (1984)
Swedish Match snuff 2004	0.32	0.027	Swedish Government Food Administration (2004)
Modern US Cigarette	Mainstream smoke, 20 cigarettes	0.05	Harris (2004)

Table 2B
Contents and estimated uptakes of NNN in humans from use of selected tobacco products.

Type of tobacco	NNN content (μg/g)	Estimated total human uptake (μg/kg bw/day)	Reference
Sudanese "Toombak"	500–3080	43–264	Idris et al. (1991)
Swedish snuff 1980	12.5	1.07	Hoffmann and Adams (1981)
Swedish snuff 1983	7.6	0.66	Österdahl and Slorach (1984)
Swedish Match snuff 2004	0.96	0.082	Swedish Government Food Administration (2004)
Modern US cigarette	Mainstream smoke, 20 cigarettes	0.07	Harris (2004)

tritiated NNK and NNN, the release of HPB upon mild hydrolysis was found to be variable (10–40%) when expressed as percentage of the *total* radioactivity in hemoglobin. However, it is important to note, that the total activity in hemoglobin reflects the generation a mixture of adducts to nucleophilic centers in globin, and which can be assumed to be characterized by different reaction kinetics; more importantly – apparently not released upon mild hydrolysis. The identity of the product generated by hydrolysis has been unequivocally identified by GC–MS as 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB) from treatment with NNK as well as with NNN (Carmella and Hecht, 1987). Further, the relation between release of HPB and administered dose is not affected by the variations in recovery expressed as percentage of total radioactivity in DNA. The dose–response was virtually strictly linear over a dose range of more than two orders of magnitude (Murphy et al.,1990; Carmella and Hecht, 1987). There is no reason to believe, that these results are not valid for human hemoglobin.

Instead of derivatization of HPB with pentafluorobenzoyl chloride after hydrolysis of DNA and subsequent extraction, the group of Hecht developed a method for the quantitative analysis of different POB–DNA adducts that involves enzymatic digestion of the purified DNA, followed by enrichment and the subsequent use of high performance liquid chromatography-electrospray ionization-tandem mass spectrometry (HPLC–ESI-MS/MS) (Lao et al., 2007).

Whereas several POB–DNA adducts have been characterized in the rodent by the Hecht group (Lao et al., 2007), not all such adducts may have been quantified by the method used by Richter et al. (2002) and Schlöbe et al. (2008). However, if so, the difference between expected and measured adduct levels in humans would be even larger.

9. Relevance of the molecular biology data

Conventional epidemiological studies involving low risk scenarios are bugged by bias and confounding, the impact of which is difficult to adjust for. The biological credibility of the epidemiological studies involving smokeless tobacco would clearly benefit from an assessment of existing relevant molecular biology data.

Cellular DNA damage that is misrepaired or not repaired, constitutes a necessary, although not sufficient prerequisite for induction of cancer. The first conclusive proof of the relationship between carcinogenesis and DNA repair capacity in humans was obtained when it was shown that skin cancer in xeroderma pigmentosum was due to a defect in the nucleotide excision DNA repair pathway (Cleaver, 1968).

Jarabek et al. (2009) claim that "DNA adducts are considered biomarkers of exposure, whereas gene mutations and chromosomal alterations are often biomarkers of early biological effects and also can be bioindicators of the carcinogenic process". Whereas the latter part of this statement is certainly correct, it is not true, that a highly pro-mutagenic adduct, such as O6-mGua can be regarded solely as an indicator of exposure. This adduct gives rise to G:C to A:T transitions, and if not repaired, may result in miscoding mutations during DNA replication (Eadie et al., 1984; Jansen et al., 1996) with the subsequent possible development of neoplasia (Peterson and Hecht, 1991; Margison et al., 2002). The linear correlation between levels of O6-mGua in lungs and lung tumor incidences in mice and rats is also noteworthy (Belinsky et al., 1990; Peterson and Hecht, 1991). In the absence of a measurable increase in early key events as represented by the induction of pro-mutagenic DNA adducts, an increase in lung tumors above background would certainly not occur.

C3HeB male mice are characterized by an exceptionally high incidence of spontaneous hepatocellular carcinoma. In the corresponding transgenic mouse strain overexpressing human O^6-methylguanine-DNA methyltransferase the incidence of carcinomas, as well as of G:C to A:T transition mutations, is drastically reduced. (Zhou et al., 2001).

Nevertheless, adduct levels in specific tissues are often *quantitatively* poorly correlated with tumor development. Other factors downstream of DNA adduct formation such as DNA repair, cytotoxicity and cell proliferation are important determinants for the appearance of tumors. Thus, while Clara cells exhibit the highest adduct levels in rat lung, the majority of lung tumors begin as proliferative changes of Type II cells with subsequent progression to adenomas and carcinomas within the hyperplastic area (Belinsky et al., 1990, 1991). From the higher levels of NNK-induced O6-mGua adducts in the respiratory mucosa as compared to the olfactory mucosa, one would expect neoplasia to develop in this region. However, malignant tumors was found to originate from the olfactory region, and appeared only after chronic treatment with high doses of NNK (50 mg/kg/day). In this case differences in levels of AGT did not offer an explanation (Belinsky et al., 1987a).

Notwithstanding these considerations, and accepting the unavoidable uncertainty due to species differences, a comparison of specific DNA adduct levels from TSNA in experimental animals with those found in humans may provide insight as to the origin of specific DNA adducts detected in humans. The observed *increment* of POB–hemoglobin adduct levels in smokers and users of snuff above the background found in non-smokers (0.05–0.5 fmol

Table 3
Comparisons between rodent adduct concentrations with those found in the corresponding tissues from humans. The calculated uptakes from Tables 2A and 2B were multiplied by the specific alkylation efficacy found in rat tissues (expressed as adducts/10^9 TN per μg NNK intake/kg and day, or fmol HPB/mg globin for Hb adducts). For DNA POB adducts, esophagus data for NNN constitute the basis, and where it is assumed that NNK in tobacco contributes an additional 25% per dose unit. (3A) O^6-methylguanine; (3B) 7-N-methylguanine; (3C) pyridyloxobutyl DNA adducts; and (3D) hydroxypyridylbutanone releasing Hb adducts.

	Adduct levels detected in humans (adducts/10^9 TN)	Alkylation efficacy, rat (adducts/10^9 TN per μg NNK/kg/day)	Expected adduct levels extrapolated from rodent data (adducts/10^9 TN)				
(A) *O6-mGua*							
Whole lung	25–380 (1)	~1.0 (2)(3)	53–674	0.16	0.28	0.027	0.07
Liver	28–168 (4)	~0.1 (2)	5–67	0.016	0.014	0.003	0.007
Leukocytes	1.8–12 (4) 11–12 (9)	No information					
Type of tobacco			Sudanese Toombak	Swedish oral snuff 1980	Swedish oral snuff 1983	Swedish Match oral snuff 2004	Modern US cigarette
(B) *7-mGua*							
Whole lung	650 (smokers; $n = 7$) 400 (non-smokers; $n = 2$) (5) 250; $n = 10/80$ samples (7)	13 (6)	689–8672	2.08	3.64	0.35	0.65
Liver		13 (6)	689–8672	2.08	3.64	0.35	0.65
Leukocytes	320 (smokers; $n = 11$) 250 (non-smokers; $n = 8$) (5)	1.3 (6)(8)	69–880	0.21	0.36	0.035	0.06
Type of tobacco			Sudanese Toombak	Swedish oral snuff 1980	Swedish oral snuff 1983	Swedish oral snuff 2004	Modern US cigarette
(C) *POB–DNA adducts (total)*							
Whole lung	Cancer patients 121 (smokers; $n = 21$) 18 (non-smokers; $n = 11$) (10) Sudden death victims 28 (smokers; $n = 32$) 18 (non-smokers; $n = 56$) Females 14 (smokers; $n = 8$) 24 (non-smokers; $n = 14$) (11)	0.25 (NNK) (6) 0.27 (NNN) (12)	25–240	0.33	0.25	0.03	0.03
Type of tobacco			Sudanese Toombak	Swedish oral snuff 1980	Swedish oral snuff 1983	Swedish oral snuff 2004	Modern US cigarette
	Adduct levels detected in humans (fmol HPB/mg globin)	Alkylation efficacy, rat (fmol HPB/mg globin)*	Expected adduct levels extrapolated from rodent data (fmol HPB/mg globin)				
(D) *POB–Hb adducts*							
	0.029 (non-smokers) 0.080 (smokers) 0.52 (users of US oral snuff of unknown purity) (13)	1.7 (NNK) (6) 0.9 (NNN) (14)	129–1386	1.24	1.07	0.12	0.15
Type of tobacco			Sudanese Toombak	Swedish oral snuff 1980	Swedish oral snuff 1983	Swedish oral snuff 2004	Modern US cigarette

(1) Wilson et al. (1989); (2) Belinsky et al. (1990); (3) Upadhyaya et al. (2009); (4) Kang et al. (1995); (5) Zhao et al. (1999); (6) Murphy et al. (1990); (7) Blömeke et al. (1996); (8) Bianchini and Wild (1994); (9) Georgiades et al. (2000); (10) Höltzle et al. (2007); (11) Schlöbe et al. (2008); (12) Lao et al. (2007); (13) Carmella et al. (1990); (14) Carmella and Hecht (1987).
* Expressed per μg NNK(NNN)/kg and adjusted for 40 days' exposure.

HPB/mg globin) – a relevant indicator of rates of activation of TSNA from tobacco – lie in a range predicted by the extrapolated experimental data (Table 3D), and provides justification for this type of extrapolation, given that comparisons are based on the same type of tissue.

Although an increased risk for cancer from the use of Sudanese Toombak is consistent with the O6-mGua levels extrapolated from rodent studies, the expected concentrations in users of modern snuff or cigarettes with respect to for this adduct (as well as for 7-mGua and POB adducts) lie about two orders of magnitude below those actually found in humans (Tables 3A–C), a discrepancy that can hardly be explained by species differences. This conclusion is valid even when the exceptionally high alkylation efficacy of a cell type like Clara cells in rat lung is used as basis for extrapolation. Surface based extrapolation that reflects metabolic rate, predicts a difference of roughly a factor 7 between rats and humans (Davidson et al., 1986). Data from humans have so far failed to reveal any significant impact from smoking on the levels of O6-mGua, which is to be expected from the rodent data.

The P450 enzymes CYP2A13, CYP2A6, and CYP2E1 are expressed in the respiratory tract, and are involved in the metabolism of NNK and NNN (Nishikawa et al. 2004; Zhu et al., 2006). CYP2A13 is particularly important in the activation of NNK (Su et al., 2000; Zhu et al., 2006), but the enzyme also metabolizes other substrates (Fukami et al., 2007), and there is considerable overlapping of substrate specificity and tissue expression between CYP2A13 and the similar CYP2A6. Experimental data (Villard et al., 1998; Nishikawa et al. 2004) as well as evidence from humans (Stepanov et al., 2008b) indicate that cigarette smoke induces the P450 enzymes involved in the metabolism of TSNA. Such induction could account for some of the observed increases in the levels of 7-mGua and POB adducts in smokers that could originate from sources other than tobacco (see below).

The great interindividual disparity found for POB-DNA adducts in humans (Hölzle et al., 2007; Schlöbe et al., 2008) agree with other findings indicating large differences in TSNA activation (Stepanov et al., 2008b). The reason why the difference between smokers and non-smokers with respect to the POB–DNA adduct concentrations was statistically not significant in sudden death victims (Schlöbe et al., 2008), while there was a 7-fold difference between cancer patient smokers and non-smokers with respect to DNA adduct levels (Hölzle et al., 2007) is obscure. The number of heavy smokers can be expected to be higher among lung cancer cases who will exhibit a higher degree of P450 enzyme activation, not only for TSNA, but also with respect to the unknown precursors that do not originate from tobacco generating POB-DNA as well as methylated DNA adducts (see Section 10). Further, the 28 times higher 7-meGua levels in macrophages from individuals

possessing a rare combination of the unfavorable genotypes *GSTM1 null/GSTT1 null* and *GSTP1 ile/ile* gives ample evidence of the impact of genotype (Lewis et al., 2004).

Compared with Caucasians, native Hawaiian smokers are at higher, and Japanese-American smokers at lower risk of lung cancer, even after adjusting for smoking history. A recent study demonstrated that exposure and detoxification of NNK cannot explain these differences (Derby et al., 2009). In the latter case, ethnically related life style factors, like diet, have to be considered. In two cohorts of Chinese cigarette smokers urinary excretion of total NNAL was significantly associated with risk of lung cancer (Yuan et al., 2009), but confounding by the aforementioned factors – that were not controlled for – are difficult to exclude, and may also explain some of the observed differences between smokers and non-smokers with respect to 7-meGua levels. A lower intake of chemoprotective agents from fruits and vegetables in smokers has been amply documented (Subar and Harlan, 1993).

The observation that the levels of POB–DNA adducts in oral mucosa were much higher in users of Swedish snuff than in smokers (Richter et al., 2009b) might be due to a more efficient retention of TSNA from direct exposure to smokeless tobacco in the buccal mucosa. However, this finding has, obviously, little bearing on risk for tumor development at this site from low nitrosamine snuffs for which no adequate evidence for induction of cancers is available. On the other hand, the risk for oral cancers is more than 2-fold higher in smokers as compared to never smokers. In a large Danish study, heavy smoking was found to be associated with an almost 6-fold increased risk for intra-oral squamous cell carcinoma (Bundgaard et al., 1995). These results cast doubt on the involvement of POB–DNA adducts in causing oral cancer, especially from Swedish "snuff" with a TSNA concentration of about ≈2 μg/g.

The mutational signature found in smoking associated lung tumors provides an ambiguous picture, but demonstrates the complex genotoxic impact from the various types of carcinogens present in tobacco smoke. The mutation spectra with G:C to T:A transversions of the *p53* suppressor gene found in several lung tumors from smokers is consistent with the genotoxic action from substances like PAHs or acrolein (Pfeifer et al., 2002; Pfeifer and Hainaut, 2003; Hecht, 2006). However, the mutational spectra were only found in a minor part of all lung tumors, where the origin of the majority with *p53* mutations is unknown. In a recent analysis of more than 22,000 somatic substitutions in a small-cell lung cancer line, G:C to T:A transversions typical of bulky adducts were most common (34%), followed by G:C to A:T (21%) and A:T to G:C (19%) transitions (Pleasance et al., 2010). Although by themselves these results do not rule out the participation of TSNA, evidence for a major role of the PAH type of compounds in smoking induced lung cancer adducts is somewhat strengthened. Whereas smoking and use of snuff result in similar exposures to the systemic carcinogens NNK and NNN, only smoking is associated with human lung cancer. This observation gives further support to the notion that TSNA probably play a minor role in the induction of smoking-related cancers.

10. The origin of methylated and POB–DNA adducts in "unexposed" humans

In this context it is important to note, that a number of recent investigations have demonstrated that background levels of promutagenic DNA adducts, obviously caused by unknown dietary or endogenous sources, are ubiquitously found in healthy humans without known exposures to either tobacco or other alkylating agents (EUROGAST, 1994; Povey, 2000a; Schlöbe et al., 2008). The results from this review provide additional support for this notion. Povey (2000a) pointed out, that DNA damage in humans may also arise from endogenous exposures.

As mentioned above, methylation of the 7 position in guanine is assumed to be much less important in the context of cancer initiation. The predicted levels for 7-mGua in tissues like liver and lung from exposure to NNK in cigarette smoke and from modern snuff are likewise insignificant when compared to those actually measured in humans. One might argue, that data from the rat used in this context were obtained after only 4 daily doses, whereas exposure in smokers is chronic. However, in view of the fast depurination of 7-mGua (Pegg and Hui, 1978), and fast repair of apurinic sites would lead to a rapid establishment of a steady state. More convincing evidence of an increased level in smokers with respect to the exposure biomarker 7-meGua was obtained for DNA extracted from bronchial lavage samples (Lewis et al., 2004). For other tissues, at most only a modest increase was found based on a very small number of cases (Mustonen and Hemminki, 1992; Mustonen et al., 1993; Szyfter et al., 1996). In addition, determinations of the 7-mGua adducts in human tissues obtained by the thin layer chromatographic methods used in most of the above mentioned postlabelling studies are difficult to interpret, because of contamination by 7-(2-hydroxyethyl)-guanine adducts (Kumar and Hemminki, 1996; Zhao et al., 1999). 7-(2-Hydroxyethyl)-guanine adducts are present at significant levels, some of which may be caused by hydroxyethylating agents like ethene and ethylene oxide in tobacco smoke (Törnqvist et al., 1986; Bolt, 1996; Hoffmann and Hoffmann, 1998). Ethylene oxide has been detected at a concentration that is about one order of magnitude higher than for NNK in mainstream tobacco smoke (Hoffmann and Hoffmann, 1998). Consequently, the difference between smokers and non-smokers decreased appreciably as the detection method improved (Zhao et al., 1999). Blömeke et al. (1996) who isolated 7-mGua prior to postlabelling failed to find any significant differences between smokers and non-smokers. Finally, tobacco smoke contains other methylating agents than NNK, such as N-nitrosodimethylamine (Tricker et al., 1991). However, the origin of the high background concentrations of 7-mGua adducts in humans not exposed to tobacco remains obscure.

Whereas Lao et al. (2007) found no POB adducts in esophageal, liver or lung DNA from control rats, the presence of high adduct levels in individuals who do not use tobacco confirm the existence of sources other than tobacco to HPB-releasing adducts (Hölzle et al., 2007; Schlöbe et al., 2008). The tobacco alkaloid myosmine, found in appreciable quantities in several basic food and dietary components (Tyroller et al., 2002), is readily nitrosated *in vitro* giving rise to HPB (Wilp et al., 2002; Zwickenpflug, 2000; Hecht et al., 2007b). For this reason myosmine has been suggested to represent an additional source of POB-adducts. Upon administration of myosmine to rats small amounts of HPB and the major metabolite 4-oxo-4-(3-pyridyl)butyric acid that has been postulated to derive from HPB, were detected in the urine, but there was no evidence for the formation of NNN (Richter et al., 2002; Zwickenpflug et al., 2005). On basis of experiments in rats given a combination of nitrite and myosmine, the efficacy of this route of conversion to NNN in the stomach has been questioned (Hecht et al., 2007b). There may exist other pathways for endogenous activation of this alkaloid. Zwickenpflug and Tyroller (2006) have described the *in vitro* generation of HPB and other products from myosmine by a mixture of hydrogen peroxide and acetic acid anhydride.

11. Conclusions

Based on DNA adduct data extrapolated from rodents, exposure to Sudanese "Toombak" can be expected to result in levels of TSNA-induced DNA lesions that are far above those found in "unexposed" individuals, implying a tangible risk for developing

cancer, in agreement with the clinical observations. The expected levels of pro-mutagenic adducts from exposure to Swedish snuff as well as from contemporary American cigarettes are, on the other hand, about two orders of magnitude lower than those actually found in the corresponding tissues in humans, a discrepancy that can hardly be explained by species differences. The high background concentrations of methylated and POB–DNA adducts in "unexposed" humans must be ascribed to other sources than tobacco. An external exposure to TSNA that does not appreciably affect the "normal" background concentrations of critical pro-mutagenic DNA adducts should be considered as "virtually safe", irrespective of the shape of the dose–response relationship.

Conflict of interest statement

This author served as international coordinator for a clinical study on smoking cessation carried out in Belgrade, Serbia, supported by Swedish Match AB, a producer of smokeless tobacco. However, no support from industry has been obtained in preparing this article.

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