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Heterocyclic aromatic amines and their contribution to the bacterial mutagenicity of the particulate phase of cigarette smoke

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HIGHLIGHTS

- A method for the quantification of 8 heterocyclic aromatic amines (HAAs) in cigarette smoke (CS) is reported.
- The mutagenic potency of these 8 HAAs and that of CS was determined in the Salmonella Reverse Mutation Assay.
- The 8 HAAs do not contribute significantly to the bacterial *in vitro* mutagenicity of CS.

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ABSTRACT

Heterocyclic aromatic amines (HAAs) rank among the strongest known mutagens. Approximately 30 HAAs have been found in cooked foods (broiled, fried, and grilled) and several HAAs have been characterized as animal carcinogens. Nine HAAs have also been reported to be constituents of cigarette smoke (CS) raising concerns that HAAs might contribute significantly to the known carcinogenicity of CS. As HAAs are found predominantly in the total particulate matter (TPM) of CS, an improved method for the quantification of HAAs in TPM is reported allowing detection and quantification of 8 HAAs in a single run. The mutagenic potency of these HAAs and that of TPM from the reference cigarette 2R4F was determined in the Salmonella Reverse Mutation Assay (Ames assay) with tester strain TA98 and a metabolic activation system. The 8 HAAs, when applied together in the Ames assay, showed a clear sub-additive response. Likewise, the combination of HAAs and TPM, if at all, gave rise to a slight sub-additive response. In both cases, however, the sub-additive response in the Ames assay was observed at HAA doses that are far above the amounts found in CS. The contribution of the individual HAAs to the total mutagenic activity of TPM was calculated and experimentally confirmed to be approximately 1% of the total mutagenic activity. Thus, HAAs do not contribute significantly to the bacterial *in vitro* mutagenicity of CS TPM.

1. Introduction

Since the detection of substances with very high bacterial mutagenicity in cooked/broiled meat and fish and their identification as heterocyclic aromatic amines (HAAs), (Commoner et al., 1978; Sugimura et al., 1977a,b) this class of compounds has initiated extensive research that is still ongoing. HAAs are formed typically at higher temperatures (140–165 °C) as the result of Maillard reactions involving creatinine, free amino acids (especially tryptophan and glutamic acid), and sugars (Wakabayashi et al., 1993). Special attention is given in the literature to the following carboline type of HAAs: MeAαC, AαC, Trp-P-1, Trp-P-2, Glu-P-1, Glu-P-2, and the aminoimidazo type of HAAs: IQ, MelQ, MelQx, PhlP. Harman and norharman, although β-carbolines, are not considered as member of the HAA class in most publications as

Abbreviations: AαC, 2-amino-9*H*-pyrido[2,3-*b*]indole (amino-α-carbolin); CI, chemical ionization; CS, cigarette smoke; DMSO, dimethyl sulfoxide; GC–MS/MS, gas chromatography tandem mass spectrometry; Glu-P-1, 2-amino-6-methyldipyrido[1,2-*α*:3′,2′-*d*]imidazole; Glu-P-2, 2-aminodipyrido[1,2-*α*:3′,2′-*d*]imidazole; ISO, International Organization for Standardization; IQ, 2-amino-3-methylimidazo[4,5-*f*]quinolone; MeAαC, 2-amino-3-methyl-9*H*-pyrido[2,3-*b*]indole (methylamino-*α*-carbolin); MelQ, 2-amino-3,4-dimethylimidazo[4,5-*f*]quinolone; MelQx, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline; MS, mainstream smoke; NADPH, nicotinamide adenine dinucleotide phosphate; NCI, negative chemical ionization; OECD, Organization for Economic Co-operation and Development; PCI, positive chemical ionization; PFAA, pentafluoro acetic acid anhydride; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; TPM, total particulate matter; Trp-P-1, 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole; Trp-P-2, 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole.

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they lack an exocyclic amine group. The exocyclic amine group of HAAs can undergo metabolic activation by *N*-hydroxylation producing an intermediate (arylnitrenium ion) which has been implicated in general toxicity and DNA damage (Turesky and Le Marchand, 2011).

With the exception of harman and norharman, the mentioned HAAs exhibit a clear *in vitro* activity inducing reverse mutations in *Salmonella typhimurium* (Ames assay), morphological transformation in mouse fibroblasts, micronucleus induction in human cells, and DNA strand breaks (Comet Assay) in human cells (Pfau et al., 1999; Dumont et al., 2010; Jagerstad and Skog, 2005; Sorensen et al., 1996; Viegas et al., 2012; Haza and Morales, 2011; Platt et al., 2010), and genotoxic effects *in vivo* as DNA adducts (Arimoto-Kobayashi et al., 2006; Dingley et al., 2003). Micronuclei formation could be demonstrated for PhIP in mice but not for MeIQ and IQ (Durling and Abramsson-Zetterberg, 2005). The carcinogenicity of PhIP, MeIQ, and IQ in mice and rats in various organs, like liver, pancreas, colon, mammary gland, and prostate is well established at doses around 10 mg/kg/day (Ohgaki et al., 1986; Schut and Snyderwine, 1999; Wakabayashi et al., 1993). Carcinogenicity studies in nonhuman primates with PhIP, MeIQ, and IQ could only demonstrate a carcinogenic action of IQ in the liver at doses of 10 and 20 mg/kg/day (Takayama et al., 2008). Differences in metabolism between rodents and primates account for the observed differences in the carcinogenic effects (Turesky, 2005). In humans, no occupational exposures to pure HAAs have been reported. However, since the detection of HAAs in food, there are concerns that their presence in food might cause tumors in men (Commoner et al., 1978; Sugimura et al., 1977a,b). Several epidemiological studies have tried to find an association between the intake of, *e.g.*, cooked meat, cooked fish, or fried potatoes and several tumor types, especially those of the colon (WCRF/AICR, 1997). These studies are mainly based on questionnaires exploring the diet of the participants. For colorectal adenomas or carcinomas there are more studies that showed an association (although not always statistically significant) than those that gave a negative result (Kim et al., 2013; Berlau et al., 2004). Associations between HAAs and tumors at other sites are in summary even more inconclusive. Considering these uncertainties, insufficient evidence exists to establish a definite conclusion on the role of HAAs in the genesis of human tumors (Alaejos et al., 2008; Santarelli et al., 2008). This conclusion is consistent with the assessments of the International Agency for Research on Cancer that has not listed any of the HAAs as a definite human carcinogen (IARC, 2015); IQ was classified as a 'probable human carcinogen' (Group 2A) and other assessed HAAs (AαC, Glu-P-1, Glu-P-2, MeAαC, MeIQ, MeIQx, Trp-P-1, Trp-P-2) as 'possible human carcinogens' (Group 2B). Comparing the doses that gave rise to a distinct tumor development in rodents and monkeys with the estimated daily oral intake of HAAs by humans shows that the estimated human exposure is more than 1000 times lower. As such, a not yet identified mechanism would be needed to explain a link between HAAs and human tumorigenicity (Wakabayashi et al., 1993).

Maillard reactions are well-known to occur in the burning cigarette and as all components necessary to form HAAs are present in tobacco, it has been suggested that HAAs should also be found in cigarette smoke. Three years after the initial studies of Sugimura et al. at the National Cancer Center Research Institute in Japan where HAAs in the diet could be identified (Sugimura et al., 1977a,b) the first HAAs, AαC and MeAαC, were identified and quantified in TPM of cigarette smoke (Yoshida and Matsumoto, 1980; Matsumoto et al., 1981). Further studies by several different laboratories using different analytical methodologies identified additional HAAs in TPM (Yamashita et al., 1986; Kanai et al., 1990; Sasaki et al., 2001a; Manabe et al., 1991, 1990; Manabe and Wada, 1990; Wakabayashi et al., 1995; Kataoka et al., 1998; Smith et al., 2004; Turesky et al., 2005; Saha et al., 2009; Zhang et al., 2011).

Despite some concerns regarding the biological activity of HAAs in TPM, it was only in 1997 that several HAAs were included in a revised list of "[c]arcinogens in tobacco and cigarette smoke" issued by Hoffmann and Hoffmann (1997). More recently, the U.S. Food and Drug Administration (FDA) has included 8 HAAs in their list of 93 'Harmful and Potentially Harmful Constituents (HPHCs) in tobacco products and tobacco smoke' (FDA, 2012). A more recent list of 39 priority toxic contents and emissions of tobacco products does not include HAAs (WHO, 2015).

As data on the mutagenicity of HAAs in the context of TPM are scarce and, regarding interactions, nearly non-existent, the research presented here was targeted to corroborate the existing potency data on single HAAs, their occurrence in TPM, their contribution to the overall mutagenicity of TPM, and their interactions with TPM or between the HAAs themselves. Hereby, an improved analytical method for the quantification of the HAAs in TPM was applied.

2. Materials and methods

2.1. Cigarettes, mainstream smoke (MS) generation and trapping

The American blended reference cigarettes 2R4F were obtained from the University of Kentucky, Kentucky Tobacco Research and Development Center (Davis and Vaught, 1990). The cigarettes were conditioned unpacked in open containers according to International Organization for Standardization (ISO) standard 3402 (ISO, 1999), *i.e.*, at least 48 h at target conditions of 22 °C ± 1 °C and a relative humidity of 60% ± 3%. MS was generated on a 20-port Borgwaldt smoking machine (RM20H, Hamburg, Germany) according to ISO Standard 3308 (ISO, 1991). In brief, puff volume, puff duration, and puff frequency were 35 ml, 2 s, and 1/min, respectively.

The yields per cigarette (means ± SE, $N = 4$) obtained under these conditions were 9.77 ± 0.04 mg TPM, 0.71 ± 0.01 mg nicotine, 1.08 ± 0.04 mg water, and 11.2 ± 0.1 mg carbon monoxide.

The TPM of 5 cigarettes/sample was trapped on a glass fiber filter (Cambridge filters; Filtrona Instruments, Milton Keynes, UK) for chemical analyses and TPM from 20 cigarettes (on 2 filters) for mutagenicity determination.

2.2. Sample preparation and chemical analyses

The analytical method used for the identification and quantification of HAAs was based on a previously published method (Sasaki et al., 2001b) with modification as follows (major differences to Sasaki et al. were triple quadrupole technology in both PCI and NCI modes instead of single quadrupole technology only in the NCI mode):

TPM trapped on glass fiber filters was extracted with 11 ml hydrochloric acid (0.1 N) containing 100 μl calibration standard solution (d_3-IQ; Toronto Research Chemicals, Inc. (Toronto, Ontario, Canada)) for 30 min in an ultrasonic bath. The extract was washed two times by shortly shaking with approximately 10 ml dichloromethane which was removed and discarded. Then the pH was adjusted by the addition of 15 ml of K_2CO_3 solution (saturated) and washed two times again with 10 ml dichloromethane, each. After each washing step, the dichloromethane extracts were concentrated under a nitrogen flow to 2 ml. The sample was derivatized for 30 min at 70 °C by addition of 20 μl pyridine and 4 μl pentafluoro acetic acid anhydride (PFAA) prior to addition of 4 μl *N,N*-dimethylformamide acetal-methyl-8 (Sigma–Aldrich, Buchs, Germany). The solution was concentrated to dryness under nitrogen and the residue dissolved in 0.1 ml