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Investigation of the genotoxic effects of 2-amino-9H-pyrido[2,3-*b*]indole in different organs of rodents and in human derived cells

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Abstract

Aim of the present study was the investigation of the genotoxicity of amino-α-carboline (AαC) in human derived cells and of its organ-specific effects in laboratory rodents. This heterocyclic amine (HA) is contained in fried meat and fish in higher concentrations than most other cooked food mutagens. In the present experiments, AαC caused dose-dependent induction of micronuclei in the human derived hepatoma cell line HepG2 at concentrations ≥50 μM. In contrast, no significant effects were seen in Hep3B, another human hepatoma cell line, which may be explained by the concurrent lower activity of sulfotransferase (SULT), an enzyme playing a key role in the activation of AαC. A positive result was also obtained in the single cell gel electrophoresis (SCGE) assay in peripheral human lymphocytes, but the effect was only significant at the highest concentration (1000 μM). In Fischer F344 rats and ICR mice, the liver was the main target organ for the formation of DNA adducts (at ≥50 mg/kg bw), and in lungs and colon substantially lower levels were detected. Identical organ specificity as in the DNA adduct measurements was seen in SCGE assays with rats, whereas in mice the most pronounced induction of DNA migration was observed in the colon. Comparison of our results with data from earlier experiments indicate that the genotoxic potency of AαC is equal to that of other HAs, which are contained in human foods in much smaller amounts. Therefore, our findings can be taken as an indication that the human health risk caused by exposure to AαC is higher than that of other HAs that are formed during the cooking of meat and fish.

Keywords: Genotoxicity; 2-Amino-9H-pyrido[2,3-*b*]indole; DNA

1. Introduction

2-Amino-9H-pyrido[2,3-*b*]indole (amino-α-carboline, AαC) has been found in fried food, mainly in beef and fish, at higher concentrations than many other heterocyclic amines (HAs) [1–3]. Dietary intake of AαC in the US population has been estimated to be 5 ng/kg per day constituting the second most abundant of five HAs examined, after PhIP [4]. However, fewer experimental data on the genotoxic and carcinogenic properties of this compound are available than for many other cooked food mutagens. AαC was mutagenic in the Salmonella/microsome assay [5–7] and induced mutations and chromosomal aberrations in CHO cells (for review see [8]) but its activity was by far lower than that of other HAs (e.g. quinolines and quinoxalines). Since these models require addition of exogenous enzyme

Abbreviations: AαC, 2-amino-9H-pyrido[2,3-*b*]indole; 4,8-DiMeIQx, 2-amino-3,4,8-trimethylimidazo-[4,5-*f*]quinoxaline; CHO, Chinese hamster ovary; DMEM, Dulbecco's Minimal Essential Medium; EROD, ethoxyresorufin *O*-deethylase; IQ, 2-amino-3-methylimidazo-[4,5-*f*]quinoline; MeIQ, 2-amino-3,4-dimethylimidazo-[4,5-*f*]quinoline; MeIQx, 2-amino-3,8-dimethylimidazo-[4,5-*f*]quinoxaline; MN, micronuclei; MROD, methoxyresorufin *O*-deethylase; NAT, *N*-acetyltransferase; PBS, phosphate buffered saline; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; SCGE, single cell gel electrophoresis; SULT, sulfotransferase; Trp-P-2, 3-amino-1-methyl-5H-pyrido[4,3-*b*]indole

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homogenates (e.g. S9-mix), they are not suitable for quantitative risk assessment. Only few data are available from studies with laboratory rodents. For example, it was shown that the compound causes DNA adduct formation in the liver of Sprague–Dawley rats [9]. Additionally, one report is available with transgenic mice (hemizygous *lac*I), in which AαC, unlike PhIP, induced mutations specifically in the colon [10]. Results of long-term carcinogenicity studies are inconsistent: Formation of liver tumors and tumors in blood vessels in CDF_1 mice was reported by a Japanese group [11] and in neonatally exposed multiple intestinal neoplasia mice, formation of colon tumors was enhanced by AαC [12]. In contrast, no indication of induction of mammary gland tumors was found in Sprague–Dawley rats [9] and in Syrian golden hamsters no adenocarcinoma formation and no hyperplasia in pancreatic ducts were seen after chronic administration of AαC [13].

Aims of the present study were (a) to clarify whether this compound causes DNA damage in human derived cells and (b) to investigate the organ-specific genotoxic effects of AαC in rodents. The extent of DNA damage was measured with the alkaline single cell gel electrophoresis assay (SCGE or comet assay). This test system, which detects DNA migration in an electric field, is highly sensitive towards DNA damage caused by HAs [14–16]. To elucidate if, and to which extent, AαC-induced DNA migration correlates with DNA adduct formation in the animals, ^{32}P post-labelling experiments were carried out. To find out if the compound is genotoxic in human derived cells, micronucleus (MN) and SCGE experiments were conducted with two human derived hepatoma cell lines (HepG2 and Hep3B) and with peripheral lymphocytes, respectively. Additionally, the activities of sulfotransferase (SULT) and cytochrome P4501A (CYP1A) were measured in the human hepatoma cell lines, two enzymes which play a key role in the activation of AαC [17–19].

2. Experimental procedures

2.1. Chemicals

2-Amino-9H-pyrido[2,3-*b*]indole (Fig. 1) was purchased from Toronto Research Chemicals (Toronto, Canada), inorganic salts for buffer solutions and dimethylsulfoxide (DMSO) came from Merck (Darmstadt, Germany), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1,4-dithio-DL-threitol (DTT), ethoxyresorufin, methoxyresorufin, resorufin, protein standard (bovine serum albumine), reduced β-nicotinamid-adenin-dinucleotid-phosphate (NADPH), 4-nitrophenol, cytochalasin B, ethidium bromide stain, trypan blue solution, proteinase K and histopaque-1077 were obtained from Sigma–Aldrich (St. Louis, MO, USA), Dulbecco's Minimal Essential Medium (DMEM), fetal calf serum (FCS) and trypsin were purchased from PAA (Linz, Austria); the protein assay was obtained from Bio-Rad Laboratories (Munich, Germany), dopamine came from Fluka (Buchs, Switzerland), [^{35}S]-3′-phosphoadenosine-5′-phosphosulfate (PAPS) was purchased at Perkin-Elmer (Wellesley, MA, USA).

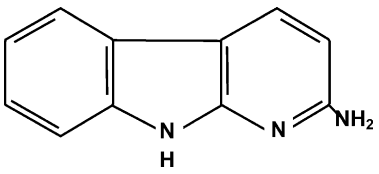

Fig. 1. Chemical structure of 2-amino-9H-pyrido[2,3-*b*]indole (AαC); CAS number: 26148-68-5.

2.2. Tests with human derived cells

2.2.1. MN test with hepatoma cell lines

HepG2 cells were kindly provided by G. Dallner (University of Stockholm, Sweden), the Hep3B cells were obtained from ATCC (Manassas, VA, USA). Deep frozen aliquots were stored in liquid nitrogen. The cells were cultivated in DMEM with 15% fetal calf serum in tissue culture flasks (200 ml, 75 cm^2 surface; Greiner Bio-One, Solingen, Germany) in CO_2-atmosphere (8% CO_2, 90% humidity, 37 °C). The MN experiments were carried out with three parallel cultures per experimental point according to the protocol of Natarajan and Darroudi [20] with minor modifications, i.e. the cells were grown in tissue culture flasks (50 ml, 25 cm^2 surface; Greiner Bio-One, Solingen, Germany). Stock solutions of the test compound were prepared with sterile DMSO and added to the cells for 24 h (final concentrations: 25, 50, 100 and 300 μM; maximum DMSO concentration 1%), the control cultures were incubated with 1% DMSO. After exposure, the cells were washed with PBS and cultivated for 26 h with cytochalasin B (final concentration 3 μg/ml in DMEM). This compound allows division of nuclei but hinders cell division, so that the cells become binucleated during mitosis and can be distinguished from mononucleated cells [21]. The cells were collected by trypsinisation (with 0.1% trypsin), treated with hypotonic solution (KCl 5.6 g/l A. bidest.) and fixed with methanol/acetic acid solution (3:1). Subsequently, dry slides were prepared and stained with Giemsa (2.5%) until the cytoplasm was visible and could clearly be distinguished from the nucleus (3–5 min). The slides were scored under a light microscope (Nikon, Microphot-FXA). Per slide, 500 binucleated cells (BNC) were evaluated (1500 cells per experimental point) at 400× magnification, MN were verified at 800× magnification. Additionally, the distribution of mono-, bi- and polynucleated cells (MNC, BNC, PNC) was monitored for each experimental point.

2.2.2. SCGE test with primary human lymphocytes

Peripheral lymphocytes were isolated from 10 ml venous blood of a healthy male donor (non-smoker, 39 years) using a separation medium with a density of 1.077 g/l (Histopaque-1077, Sigma, St. Louis, USA) according to

the protocol of Duthie et al. [22]. The cells were suspended in PBS buffer solution. Per experimental point, 0.3–0.5 × 10^6 cells were exposed for 30 min at 37 °C to different concentrations of AαC (100, 500 and 1000 μM; three parallel cultures per concentration). An identical exposure protocol was used in earlier experiments with other HAs by Anderson et al. [23]. After incubation, the viability of the cells was determined with trypan blue [24]. All cultures had a viability >90% (data not shown). The cells were washed with PBS and mixed with 50 μl of low melting agarose (0.5% in PBS) at 37 °C and placed on microscopic slides, which had been covered with a thin layer of 0.5% normal melting agarose. Lysis, electrophoresis and staining were carried out as described by Singh et al. [25]. According to the guidelines for in vitro SCGE experiments [26], three cultures were treated and from each, 50 cells were analysed for DNA migration by use of a computer aided analysis system [27].

2.3. Animal experiments

2.3.1. Single cell gel electrophoresis assay (SCGE)

Fischer F344 rats (150 ± 15 g) were purchased from Charles River Inc. (Borchen, Germany) and kept under controlled conditions (24 ± 1 °C, 50 ± 5% humidity, 12 h light cycle; three animals per cage). The experiments were started after one week of acclimation. AαC was suspended in corn oil and administered by gavage at two concentrations (50 and 100 mg/kg bw; ca. 0.2 ml per animal). The control group received the vehicle only. After 4 h, the animals were killed by cervical dislocation and the organs removed.

Male ICR mice (28 ± 2 g) were obtained from Japan SLC (Shizuoka, Japan) and an identical experimental protocol was used as for the experiments with the F344 rats, except that the mice were housed in groups of four individuals and received 0.1 ml of the test solution by gavage. Histopathological examination was carried out in order to exclude DNA migration resulting from cytotoxic effects.

The SCGE experiments were carried out according to the protocol of Sasaki et al. [28]. Four animals were used per experimental group, three slides were prepared from each organ and 50 nuclei were evaluated per slide.

2.3.2. Determination of DNA adducts

DNA was isolated from cell pellets or organs by the phenol extraction procedure according to Gupta [29] with modifications as described by Pfau et al. [30]. DNA was precipitated with ethanol/sodium chloride at −20 °C, re-dissolved in sodium citrate (0.15 mmol/l)/sodium chloride (1.5 mmol/l) buffer and quantified by UV absorbance at 260 nm.

Aliquots of the DNA (5 μg) were analysed at least in duplicate by the ^{32}P post-labelling method using SPE-extraction according to published protocols [30,31]. DNA adducts were separated by multidirectional ion-exchange TLC on polyethylene imine cellulose (Macherey und Nagel, Germany) using elution buffers [30].

Adduct levels were determined by Cerencov counting of excised adduct spots as described by Pfau et al. [30] taking into account the specific activity of the [γ-^{32}P]-ATP batch used in the experiment.

2.4. Enzyme measurements

2.4.1. Preparation of cytosols and microsomal fractions

HepG2 and Hep3B cells were grown in tissue culture flasks (162 cm^2 surface, Greiner Bio-One, Solingen, Germany) in three parallel cultures to a density of approximately 5 × 10^7 cells per flask. The cells were collected by trypsinisation (0.1% trypsin), washed in PBS and subsequently re-suspended in buffer (5 mM HEPES, 250 mM sucrose, 1 mM DTT; pH 7.4) and sonicated on ice (Branson sonifier 250; three boosts of 10 s with 30 s intervals). Cytosols were prepared by two centrifugation steps. The first was conducted at 16,000 × *g* (10 min at 4 °C; Sigma 3K30, Sigma Laborzentrifugen, Osterode am Harz, Germany). The supernatant was subjected to the second step at 100,000 × *g* (60 min at 4 °C; Beckmann Optima LE-80K Ultracentrifuge, Beckmann, Germany). The resulting supernatant (cytosolic fraction) was aliquoted and stored at −70 °C; the microsomal pellet was re-suspended in HEPES-sucrose buffer (see above) and also aliquoted and stored at −70 °C. The protein contents were determined according to Bradford [32].

2.4.2. SULT1A1 and SULT1A3 measurement

Sulfotransferase activities were measured by a modification of the methods of Foldes and Meek [33], and Anderson and Weinshilboum [34] based on the sulfate conjugation of substrates of SULT1A1 (4 μM 4-nitrophenol) and SULT1A3 (2 μM dopamin) in presence of [^{35}S]-PAPS.

2.4.3. CYP1A1 and CYP1A2 determination

CYP1A1-associated ethoxyresorufin *O*-deethylase (EROD) and CYP1A2-associated methoxyresorufin *O*-deethylase (MROD) were measured spectrofluorometrically by monitoring the formation of resorufin according to Burke et al. [35]. Catalytic activities were calculated from a standard curve of resorufin (0–90 pmol/ml).

2.5. Statistics

The results of the genotoxicity experiments were analysed with ANOVA following Dunnett's multiple comparison test. The enzyme activities in the two cell lines were compared using Student's *t*-test. *P*-values <0.05 were considered as significant.

3. Results

3.1. Micronucleus formation in the human derived cell lines

In HepG2 cells, significant MN induction was seen at all concentrations >25 μM. At the highest concentration, a

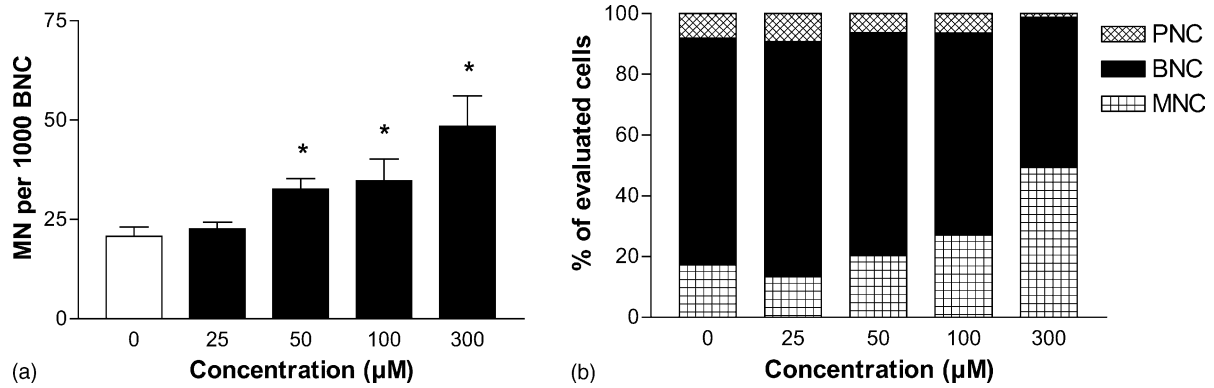

Fig. 2. Effect of AαC on induction of MN (a) and on the distribution of mono-, bi- and polynucleated cells (MNC, BNC, PNC) (b) in HepG2 cells. The cells were exposed to AαC for 24 h. Each bar represents the means ± S.D. of three parallel cultures and 1500 cells were evaluated per treatment group. (*) significantly different from control (Dunnett's test, $P < 0.05$).

2.3-fold increase over the controls was observed (Fig. 2a). The effect of AαC on mitotic activity is shown in Fig. 2b. At concentrations ≥100 μM, a decline of BNC was found; at the highest dose the amount of BNC was reduced to 49%. In contrast, no significant increase of the MN frequency was seen in the Hep3B cells (Fig. 3a); also the ratio of MNC to BNC was not affected (3b).

3.1.1. DNA migration in peripheral human lymphocytes

The results of a representative SCGE experiment with peripheral lymphocytes are shown in Fig. 4. Only marginal induction of DNA migration was observed, which was statistically significant at the highest dose level (1000 μM). The viability of the cells was not affected under any condition of test.

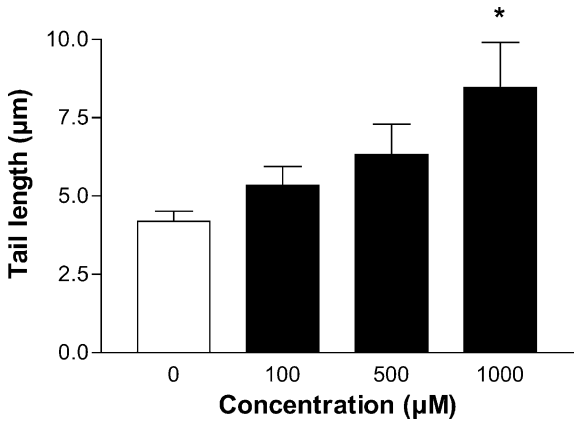

Fig. 4. Effect of AαC on DNA migration in peripheral human lymphocytes. The cells were exposed to different concentrations of the test compound for 30 min. Bars indicate means ± S.D. of data obtained with three individual cultures. (*) significantly different from control (Dunnett's test, $P < 0.05$).

3.1.2. DNA migration and adduct formation in different organs of mice

AαC caused induction of DNA migration in all three organs tested (colon, liver, lung) in a dose-dependent manner (Fig. 5a). At the highest dose level (100 mg/kg bw), the extent of DNA damage was significantly increased. The effect in the colon was approximately two-fold stronger than in the other organs. A different pattern of organ specificity was observed in the adduct measurements (5b), where the strongest effect occurred in the liver.

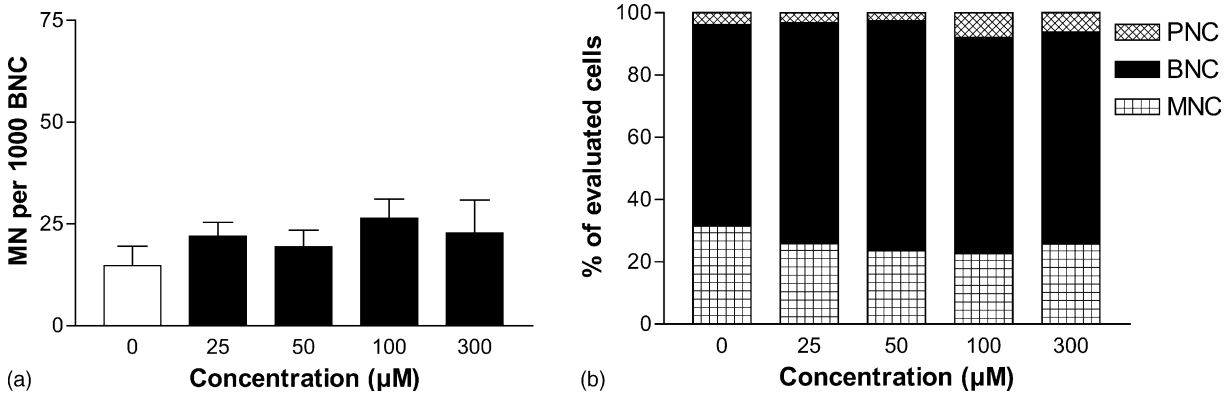

Fig. 3. Effect of AαC on induction of MN (a) and on BNC formation (b) in Hep3B cells. The experimental conditions were identical as in the experiments with HepG2 cells and details are described in the legends to Fig. 2.

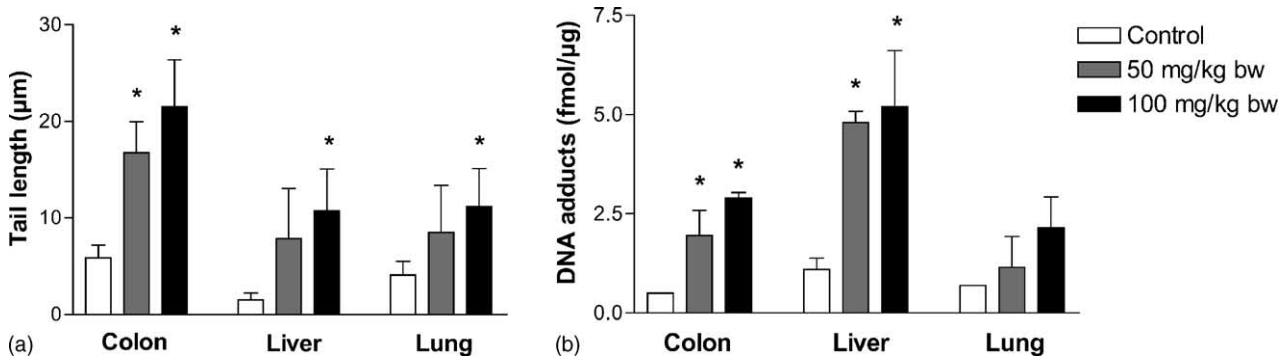

Fig. 5. Induction of DNA migration (a) and adduct formation (b) in different organs of ICR mice caused by AαC. Adduct levels are given as total adducts including background that was also observed in solvent treated animals. Bars indicate means ± S.D. of data obtained with four slides per organ. (*) significant difference from the control group (Dunnett's test, $P < 0.05$).

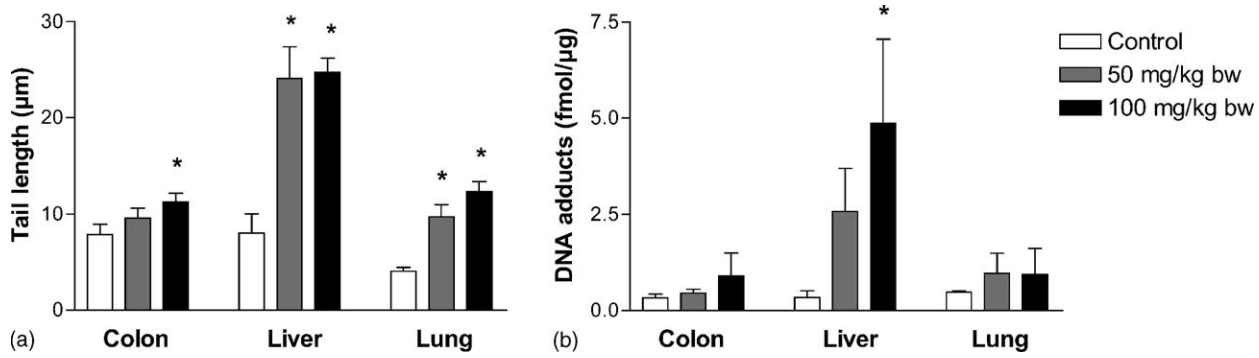

Fig. 6. Induction of DNA migration (a) and adduct formation (b) in different organs of F344 rats caused by AαC. Details are given in the legend to Fig. 5.

3.1.3. DNA migration and adduct formation in different organs of rats

The results obtained in SCGE experiments and adduct measurements with rats are shown in Fig. 6a and b. As in mice, significant induction of DNA migration was observed in all organs, but in contrast, the strongest extent of DNA damage was measured in the hepatic tissue. With 50 mg/kg, the average tail length was approximately three-fold higher than the background level. As far as the DNA adduct measurements are concerned, there was no species difference in organ specificity, but the absolute adduct levels in rats were lower than in mice.

3.1.4. Enzyme activities in the human derived hepatoma cell lines

A comparison of the activities of CYP1A and SULT enzymes is given in Table 1. EROD was 2.1-fold higher in the Hep3B cells than in HepG2; the activity of MROD was in both cell lines below the detection limit. The activities of two sulfotransferases were higher in the HepG2 cells, i.e. SULT1A1 was 2.3-fold higher and SULT1A3 6.4-fold

Table 1
Comparison of monooxygenase and sulfotransferase activities in HepG2 and Hep3B cells[a]

Activity	Enzyme represented	HepG2 cell line	Hep3B cell line
EROD (pmol/(min mg protein))	CYP1A1	0.17 ± 0.09	0.36 ± 0.17
MROD (pmol/(min mg protein))	CYP1A2	n.d.[b]	n.d.[b]
Sulfation of 4-nitrophenol (pmol/(min mg protein))	SULT1A1	13.6 ± 5.3	5.8 ± 5.8
Sulfation of dopamin (pmol/(min mg protein))	SULT1A3	19.5 ± 9.8[c]	3.0 ± 1.7[c]

[a] Ethoxyresorufin *O*-deethylase and methoxyresorufin *O*-deethylase were measured spectrofluorometrically in microsomal preparations according to Burke et al. [35]. Sulfotransferases were measured in cell cytosols as described by Foldes and Meek [33]; the protein levels in the preparations were determined according to Bradford [32]. The values represent means and S.D. of three separate cultures.

[b] n.d.: not detectable.

[c] Indicates significant difference between the two cell lines (Student's *t*-test, $P < 0.05$).

higher than in Hep3B, the latter being statistically significant ($P < 0.05$).

4. Discussion

The results of the present paper show clearly that AαC is genotoxic in the human derived hepatoma cell line HepG2 and also in peripheral human lymphocytes (Fig. 4). We have tested other HAs in the HepG2 cells in earlier experiments and found similar genotoxic potencies with IQ, MeIQ, MeIQx and PhIP [36]. In contrast, AαC was substantially weaker than PhIP, IQ, 4,8-DiMeIQx and 8-MeIQx in MN assays with MCL-5 cells [7]. The reason for this discrepancy might be that the human lymphoblastoid MCL-5 line is genetically engineered and expresses high levels of various cytochrom P-450 enzymes only [37], whereas HepG2 cells have retained the activities of many different xenobiotic drug metabolizing enzymes, including those involved in the activation and detoxification of HAs (for review see [38]). King et al. [19] proposed that sulfotransferase and *N*-acetyltransferase (NAT) play a key role in the activation of AαC to DNA-reactive metabolites. However, Glatt and co-workers [39,40] recently showed by use of transformed V79 cells, which constitutively expressed CYP1A2 and human sulfotransferases, that AαC is converted to genotoxic metabolites whereas negligible effects were observed in lines co-expressing CYP1A2 and NAT. The involvement of SULT in the activation of AαC might also explain the fact that only marginal (statistically not significant) genotoxicity was seen in the Hep3B line, which possesses substantially lower SULT activities than HepG2 (see Table 1). MROD was below the detection limit in both cell lines and EROD was even more active in the Hep3B cells. These cytochromes catalyse *N*-hydroxylation which is the first step in the activation of HAs, including AαC [17,18]. In the SCGE experiments with peripheral lymphocytes, only moderate, albeit statistically significant induction of DNA migration was measured. In addition, a second experiment with cells from another donor was performed and again only weak effects were found, which were statistically significant at 100 μM (2.1-fold increase over the control). We have recently tested a number of other HAs in the same experimental model and found marginal effects also with IQ, MeIQx and PhIP [41]. Anderson et al. [23] used two HAs (IQ and Trp-P-2) at similar concentrations in comet assays with primary human lymphocytes and also found weak effects with IQ. It is notable that these concentrations are much higher than the ones expected in blood serum of humans consuming HA-containing food. The lack of sensitivity of the peripheral blood cells towards HAs is probably due to the low expression of enzymes involved in the activation of HAs [42].

The results of the SCGE experiments and DNA adduct measurements with rodents show that the genotoxic effects of AαC are highly organ-specific. In the F344 rats, the maximum adduct levels and DNA migration were detected in the liver. Notably, this compound also induced preneoplastic lesions (GST-P$^+$ foci) in rat liver [43]. The organ specificity of the genotoxic effects of AαC in rats (Fig. 6) can be explained by the distribution of sulfotransferases, which are expressed to a substantially higher extent in the liver than in other organs [44–46]. Also in mice, the liver was the main target organ for adduct formation (Fig. 5b), whereas DNA migration was higher in the colon (Fig. 5a). As mentioned above, earlier studies indicated that the colon is a target organ in mice for tumor induction by AαC [10,12]. The discrepancy between adduct and comet formation in mice might be due to differences in DNA-repair processes in different organs. In earlier experiments we tested two other HAs, namely IQ and PhIP, in SCGE assays with F344 rats under identical experimental conditions as AαC. PhIP induced less DNA migration than the carboline in both, colon and liver, whereas IQ caused a similar effect as AαC in the liver and more pronounced DNA damage in the colon [16,47]. Also from experiments with mice, results with different HAs are available [48,49]. Again, the genotoxic potencies of quinoline and quinoxaline compounds were similar to those observed with AαC in the present study.

The most important observations of the present experiments are that (a) AαC is an equally potent genotoxin as other HAs in human derived cells and also in rodents and (b) that it causes organ and species-specific effects which differ from those seen with other cooked food mutagens. As mentioned above, the average intake of AαC by consumption of cooked meats and fish in the Western diet is substantially higher than that of other HAs, except PhIP (e.g. 2-fold higher than that of MeIQx, 6-fold higher than DiMeIQx and 18-fold higher than IQ [4]). Therefore, our findings support the assumption that the cumulative exposure to AαC may constitute a higher human health risk than that caused by most other HAs.

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