Hemoglobin adducts in workers exposed to benzidine and azo dyes

Armin Beyerbach, Nathaniel Rothman, Vijai K. Bhatnagar, Rekha Kashyap and Gabriele Sabbioni

1Institute of Environmental and Occupational Toxicology, Casella Postale 108, CH-6780 Airolo, Switzerland. 2Occupational and Environmental Epidemiology Branch, Division of Cancer Epidemiology and Genetics, National Cancer Institute, National Institutes of Health, Department of Health and Human Services, Bethesda, MD, 20892, USA. 3National Institute of Occupational Health, Ahmedabad, India and 4Walther-Straub-Institut für Pharmakologie und Toxikologie, Ludwig-Maximilians-Universität München, Nussbaumstrasse 26, 80336 München, Germany

Abbreviations: 3ABP, 3-aminobiphenyl; 4ABP, 4-aminobiphenyl; AcBz, N-acetylbenzidine; Ac4ABP, N-acetyl-4-aminobiphenyl; AcMDA, N-acetyl-4,4′-methylenedianiline; Bz, Benzidine; DcBz, 3,3′-dichlorobenzidine; DMOBz, 3,3′-dimethoxybenzidine; EI, electron-impact ionization; ELCR, excess lifetime cancer risk; Hb, hemoglobin; HERP index, human exposure rodent potency index; HFBA, heptafluorobutyric anhydride; 2MA, 2-methylaniline; 4MA, 4-methylaniline; MDA, 4,4′-methylenedianiline; NAT2, N-acetyltransferase 2; NCI, negative chemical ionization; PFPDA, pentafluoropropionic anhydride.

Introduction

Benzidine (Bz) is a known human carcinogen (1), and several azo dyes contain Bz, which can be metabolically released (2). Several epidemiological studies indicate that N-acetyltransferase 2 (NAT2)-related slow N-acetylation increases bladder cancer risk among workers exposed to arylamines, presumably because N-acetylation is important for the detoxification of these compounds (3). Hayes et al. (4) showed that NAT2 slow acetylation phenotype or genotype was not associated with increased risk of bladder cancer risk among Chinese workers exposed exclusively to Bz, suggesting that NAT2 N-acetylation is not a critical detoxifying pathway for this aromatic amine. A recent report that followed up this cohort and identified additional cases reported identical findings, and a pooled analysis of all data from the cohort found that NAT2 slow acetylation was associated with decreased risk of bladder cancer (5). In addition, the NAT1*10 allele was associated with a non-significant increased risk of bladder cancer in this population (5). In addition, Zenser et al. (6) showed with human recombinant NAT1 and NAT2 and human liver slices that Bz or N-acetylbenezidine (AcBz) is a better substrate for NAT1 than NAT2. Of the polymorphic NAT1 forms, higher average acetylation ratio was observed in human liver slices possessing the NAT1*10 compared with the NAT1*4 allele. To evaluate the biological plausibility of these findings, Rothman et al. (7–11) carried out a cross-sectional study of 33 workers exposed to Bz and Bz dyes and 15 unexposed controls in Ahmedabad, India. The presence of Bz-related DNA adducts in exfoliated urothelial cells and lymphocytes, the excretion pattern of Bz metabolites and the impact of NAT2 and NAT1 activity on these outcomes were investigated (7). Four DNA adducts were significantly elevated in exposed workers compared with controls; of these, the predominant adduct co-chromatographed with a synthetic N-(3′-phosphodeoxyguanosin-8-yl)-N′-acetylbenzidine standard and was the only adduct that was significantly associated with total Bz urinary metabolites. The predominant adduct formed was N-acetylated, supporting the concept that monofunctional acetylation is an activation, rather than a detoxification, step for Bz. At the same time, almost all Bz-related metabolites measured in the urine of exposed workers were either mono- or diacetylated among slow, as well as rapid, acetylators (95 ± 1.9 versus 97 ± 1.6%, respectively).

Results from studies of NAT2 genotype and bladder cancer risk among workers exposed to Bz or Bz-based dyes could be difficult to interpret if workers are exposed to other arylamines through metabolic release or environmental contamination. Therefore, we determined the exposure profile of carcinogenic arylamines in the present group of Bz and dye workers. We evaluated the presence of hemoglobin (Hb) adducts of 4-aminobiphenyl (4ABP), aniline, 2-methylene (2MA), 3-aminobiphenyl (3ABP), 4,4′-methylenedianiline (MDA), N-acetyl-4,4′-methylenedianiline (AcMDA),...
3,3'-dichlorobenzidine (DCBz), 3,3'-dimethylbenzidine (DMBz) and 3,3'-dimethoxybenzidine (DMOBr). The presence of Hb adducts would show that the putative carcinogenic intermediate, N-hydroxyarlylamine, was biologically available. We were particularly interested in 4ABP, as experimental studies have indicated that 4ABP can be metabolically released from Bz and azo dyes (12–15). The demonstration of 4ABP adducts in this study population would also have important implications for understanding the role that genetic variants in NAT1 and NAT2 play in Bz carcinogenesis, as N-acetylation detoxifies 4ABP and activates Bz, and could help to further understand previous studies of genetic susceptibility for Bz-induced bladder cancer.

Materials and methods

The chemicals used and the syntheses of the deuterated standards have been published previously (16,17)

Determination of Hb adducts

In this paper we present a comparison of two methods to determine the Hb-adduct levels of aromatic amines with different polarities, resulting in a two-step analytical method. The obtained red blood cells (2 ml) were lysed by addition of water (8 ml). Cell debris was removed by centrifugation. Hb was precipitated with ethanol from lysed red blood cells. The precipitated Hb was washed with ethanol-water (8:2), ethanol, ethanol-diethyl ether (3:1) and diethyl ether. The yielded Hb was dried in a desiccator and stored at −20°C until hydrolysis. The dried Hb (100 mg) was dissolved in 0.1 M NaOH (3 ml) in 25 ml tubes. An ethyl acetate solution (20 μl) containing 2 ng d5-aniline and d2-2MA, and 200 pg d4-AcBz, and 10 μl of an ethyl acetate solution containing 1 ng of d4-Bz, d2-AcBz, d2-MA, d4-DMBz and d4-DMBz were added as internal standards to the basic Hb solution (pH > 12). After 1 h in a shaking bath at 30°C the hexane (6 ml) was added. The mixture was vortexed for 1 min, frozen in liquid nitrogen and centrifuged for 5 min at 3000 g. The thawed organic layer was passed through a pipette filled with 1 g of anhydrous sodium sulfate. The sodium sulfate was rinsed with 1 ml of hexane. The dried organic phase was collected in a tube and pentafluoropropionic anhydride (PFPA) (5 μl) was added. After 10 min at room temperature the organic phase was evaporated to a volume of 0.2 ml under a gentle stream of nitrogen at 30°C in ca. 20 min. The residue was then transferred into a microinsert (200 μl) for 12 × 32 mm autosampler vials and evaporated very carefully under a stream of nitrogen at 25°C. At the disappearance of the last drop the stream of nitrogen was stopped. The residue was taken up in ethyl acetate (15 μl). The methanolic solution (10 μl) containing 24 μg of 4-methylaniline (4MA) was added to the remaining basic Hb solution. 4MA is added in order to avoid the formation of Bz-(HFBa); by transacylation of AcBz with heptfluorobutyric anhydride (HFBa) (18). A 6 ml volume of dichloromethane was added, and then the mixture was vortexed for 1 min, frozen in liquid nitrogen and centrifuged for 5 min at 3000 g. The thawed organic layer was passed through a pipette filled with 1 g of anhydrous sodium sulfate. The sodium sulfate was rinsed with 1 ml of dichloromethane. The collected organic phase was treated with HFBA (5 μl). After 10 min at room temperature 24 μg of 4MA in 10 μl of methanol were added. The solutions were dried using a speed vacuum centrifuge (Speed-Vac; Uniqueqip, Martinsried, Germany). The residue was transferred into microinserts with 2 × 75 μl of ethyl acetate and dried again. The final extracts were taken up in ethyl acetate (15 μl).

GC–MS analysis

GC–MS analyses were performed on a Hewlett-Packard gas chromatograph (HP 5890II) equipped with an autosampler (HP 7673) and interfaced to a mass spectrometer (HP 5989A). The PFPA derivatives of the arylamines were analyzed by splitless injection (2 μl) onto a fused silica column DB 1701 (J and W Scientific, Folsom, CA, USA; 15 m × 0.25 mm internal diameter with a film thickness of 1 μm) fitted with a methyl silyl deactivated retention gap (Analyt, Mülheim, Germany; 1.5 m × 0.25 mm, methyl silyl deactivated). The injection was set at 370°C and the transfer line temperature at 380°C. The GC oven temperature was held at 80°C for 0.5 min, and then increased with 50°C/min to 300°C and held for 3 min. The EI mode the electron energy was 70 eV and the ion source temperature was 200°C. For negative chemical ionization (NCI) using methane as reagent gas the electron energy was 200 eV, the emission current was 200 μA and the source temperature was 200°C. The following [M–20]+ mass fragments were monitored with a dwell time of 60 ms: m/z = 295 for 4ABP; m/z = 304 for d5-4ABP, and with a dwell time of 30 ms: m/z = 219 for aniline; m/z = 224 for d5-aniline; m/z = 233 for 2MA; and m/z = 237 for d4-2MA. The retention times for aniline, d5-aniline, 2MA, d4-2MA, 4ABP and d4-4ABP were 4.00, 4.00, 4.04, 4.04, 7.73, 7.63 and 7.64 min, respectively.

The HFBA derivatives of the arylamines were analyzed by splitless injection (1 μl) onto a fused silica column Rtx-5MS (Restek, Bad Soden, Germany; 5 m × 0.25 mm, 0.25 μm film thickness) fitted with a retention gap (Analyt, Mülheim, Germany; 1.5 m × 0.25 mm, methyl silyl deactivated). The injector was set at 370°C and the transfer line temperature at 380°C. The GC oven temperature was held at 80°C for 0.5 min, and then increased with 50°C/min to 200°C, held for 1 min and then heated at 50°C/min to 320°C and held for 1.5 min. Helium was used as carrier gas with a flow rate of 1.5 ml/min. In the NCI mode, using methane as reagent gas, the electron energy was 200 eV and the ion source temperature was 250°C. The following [M+20]+ mass fragments were monitored with a dwell time of 60 ms: m/z = 295 for 4ABP; m/z = 304 for d5-4ABP, and with a dwell time of 30 ms: m/z = 219 for aniline; m/z = 224 for d5-aniline; m/z = 233 for 2MA; and m/z = 237 for d4-2MA. The retention times for aniline, d5-aniline, 2MA, d4-2MA, 4ABP and d4-4ABP were 4.00, 4.00, 4.04, 4.04, 7.73, 7.63 and 7.64 min, respectively.

The chemicals used and the syntheses of the deuterated standards have been published previously (16,17)
Bz, dye and control workers were 26.5 ± 5.1, 24.1 ± 3.6 and 23.3 ± 3.1, respectively. The mean (±SD) years that the Bz, dye and control workers worked in their current factory were 3.2 ± 1.3, 3.5 ± 1.6 and 5.7 ± 3.4, respectively. The factories that manufactured compounds Bz and Bz-based dyes were dusty, and workers had extensive dermal and respiratory contact with these compounds. Bz and Bz-dye manufacturing were banned in India in 1994, and these factories were subsequently closed.

**Statistical analyses**

Statistical analyses were performed with the program SPSS 10.0. The Hb-adduct levels of the exposed workers (n = 33) were not normally distributed, except for aniline (One-Sample Kolmogorov–Smirnov Test, P < 0.05). Normal distribution was achieved after logarithmic transformation of the data (P > 0.05).

**Results**

**Hb adducts**

The PFPA and HFBA derivatives of the arylamines were analyzed by GC–MS in the NCI mode using methane as reagent gas. Bz, AcBz, 4ABP, aniline, 3ABP, 2MA, DMBz, DMOBz, MDA and AcMDA were quantified against the deuterated standards, using calibration lines that were run with each batch. The results of the positive samples are summarized in Table I. AcBz, 4ABP and aniline were found in all exposed workers (n = 33). Bz was detected in 28 exposed workers. In the controls only Hb adducts of 4ABP and aniline were found. 3ABP, DcBz, DMBz, DMOBz, MDA, and AcMDA were not found in control and exposed workers. 2MA was present at the levels found in other populations (19) but it was not work-related.

The samples with the PFPA derivatives of the arylamines were analyzed in the EI mode under the same GC conditions as in the NCI mode. The identity of the compounds aniline and 4ABP were confirmed by monitoring the main single ions. The ratio of the main mass fragments above m/z 100 of aniline (m/z = 239 and 120) and 4ABP (m/z = 315 and 168) was identical in the standards and samples.

In the present group of workers exposed to azo dyes and Bz, the 4ABP and aniline adducts were quantitatively the major adducts (Table I). For all exposed workers, the Hb-adduct levels of Bz and AcBz together were ca. 3 times lower compared with the 4ABP levels. Among all exposed workers, the Hb-adduct levels of AcBz, Bz and 4ABP correlated strongly with each other at r ≥ 0.89 (Figure 1, Table II). Aniline levels correlated with 4ABP, Bz and AcBz with r ≥ 0.62 (Table II).

In a further analysis, workers were split into Bz workers and dye workers. All Hb-adduct levels were significantly higher (Table I) in the Bz workers than in the dye workers (Mann–Whitney test or independent t-test, P < 0.01). The mean adduct levels of 4ABP, Bz, AcBz and aniline were 11, 10, 17 and 3-fold higher in Bz workers, respectively. The correlations between Bz and AcBz and between 4ABP and AcBz were very similar in both worker groups (Table II). However, in contrast to the dye workers, the correlation between 4ABP and AcBz was substantially weaker and not significant among the Bz workers. Aniline correlated with the other amines in the dye workers but not in the Bz workers.

**Urinary metabolites**

Urine samples were analyzed for base-extractable urinary metabolites. Urine metabolites of Bz have been determined and published previously (7). Urine was treated under basic conditions in the presence of the recovery standards d9-4ABP and d9-4Ac4ABP. The hexane extracts were concentrated and then analyzed by GC–MS in the EI mode. 4ABP was found in 30 of 33 urine from exposed workers and in one of the 13 control workers. The mean level of 4ABP in the exposed workers was 109 pmol/ml (16.5 = 25th, 40.8 = 50th and 169 = 75th percentile). In the sample, which belonged to the person with the highest 4ABP level, Ac4ABP was found with 79.5 pmol/ml urine. In the other 46 urine samples no Ac4ABP could be detected. The log-transformed values of 4ABP found in urine correlate the best with the Hb adducts of 4ABP, but also with the Hb adducts of AcBz, Bz and aniline (Table II). In Bz workers the median levels of 4ABP (57 versus 29.3 pmol/l) were significantly higher than in the dye workers (Mann–Whitney test, P < 0.01). Significant correlations between urine levels of 4ABP and Hb adducts were found in the dye workers, but not in the Bz workers.

**Discussion**

**Adduct levels**

This is the first study to report Hb adducts of Bz in humans. Hb adducts of AcBz were present to a much larger extent than Bz as Hb adducts. This corresponds to the metabolite distribution in urine and the DNA adducts (7). Aniline adducts were detected as well, and aniline was identified as being used in the manufacturing process of Bz dyes in these factories though it is unknown if it was present in the Bz factories. Surprisingly,
there were also very high levels of 4ABP adducts among both Bz workers and dye workers. Adducts of 4ABP have been reported in several studies dealing with smokers and non-smokers (19, reviewed in ref. 20). Adducts of aniline have been found in environmentally exposed people (19) and in workers of a rubber factory (21). The adduct level of Bz found in the present study are among the highest adduct levels of arylamines reported in the literature (20, short summary Table III). The 4ABP levels found in the present study are approximately 2000 times higher than in smokers (reviewed in ref. 20). The aniline levels are similar to the levels found in workers of a rubber factory with a higher incidence of bladder cancer (21).

Bz and 4ABP are the arylamines with the highest carcinogenic potential of the tested arylamines (Table III). Both compounds have been classified as human carcinogens (1). The presence of 4ABP in Bz-dyes and azo-dyes-exposed people might interfere with the interpretation of epidemiological studies about bladder cancer and N-acetylator phenotype. N-acetylation is a detoxification step for 4ABP, and NAT2 slow acetylation has been found to be a consistent risk factor for bladder cancer in workers (23) and the general population (24). In the case of Bz exposure, N-acetylation yielding AcBz is not a detoxification, since this is a needed step for the formation of DNA adducts. NAT2 acetylation and bladder cancer risk among workers exposed to Bz in China found that the slow acetylation phenotype was protective (5). Our data in the current report suggest that evaluation of the literature on susceptibility for Bz-induced bladder cancer and future studies need to consider the possibility that workers exposed primarily to Bz may have concurrent exposure to 4ABP or other arylamines either from environmental contamination or from endogenous release.

**Table II.** Pearson correlation of the log-transformed Hb-adduct levels of AcBz, Bz and 4ABP with the urine levels of 4ABP among workers exposed to Bz and azo-dyes

<table>
<thead>
<tr>
<th>Hb adducts</th>
<th>4ABP</th>
<th>AcBz</th>
<th>Bz</th>
<th>Aniline</th>
</tr>
</thead>
<tbody>
<tr>
<td>AcBz</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All workers</td>
<td>0.89</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dye workers</td>
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<td></td>
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<tr>
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<td>Bz</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All workers</td>
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<td>0.93</td>
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<td></td>
</tr>
<tr>
<td>Dye workers</td>
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<td>0.79</td>
<td></td>
<td></td>
</tr>
<tr>
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<td></td>
<td></td>
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<tr>
<td>Aniline</td>
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<td></td>
</tr>
<tr>
<td>All workers</td>
<td>0.68</td>
<td>0.65</td>
<td>0.62</td>
<td></td>
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<tr>
<td>Dye workers</td>
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<tr>
<td>Bz workers</td>
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<td>−0.17b</td>
<td>−0.13b</td>
<td></td>
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<tr>
<td>U-4ABP</td>
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<td></td>
<td></td>
<td></td>
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<tr>
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<tr>
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<td>0.47a</td>
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<td>Bz workers</td>
<td>0.49b</td>
<td>0.18b</td>
<td>0.35b</td>
<td>0.26b</td>
</tr>
</tbody>
</table>

For all correlations the two-tailed significance level was $P < 0.01$, except for $^aP < 0.05$ and $^b$with $P > 0.05$.

Fig. 1. Correlation of the Hb adducts found in workers exposed to Bz and azo dyes ($P < 0.01$).

**Origin of 4ABP**

Experimental studies have shown that 4ABP can be metabolically released from Bz and Bz products (12–15) and aniline can be metabolically released from azo dyes that contain aniline (e.g. Direct Black 38, Direct Green 1). Aniline has been shown to be released from azo dyes (25,26) but not from Bz. Aniline was identified as a material used in these factories to manufacture Bz dyes, but its presence in Bz factories is unknown. The factories did not record the use of 4ABP in any of these manufacturing processes. Measurement of worker exposure to 4ABP and aniline in these workplaces was not carried out. There were substantial levels of 4ABP adducts in dye workers and even higher levels among Bz workers. We attempted to explore the extent to which these may have derived from exogenous exposure versus endogenous release by considering the ratio (Table I) of the Hb adducts in the different group of workers, the correlations between adducts (Table II) and by comparison with data obtained in rat experiments (Table III). The mean ratio of AcBz/Bz was similar in Bz workers and dye workers. The mean ratios of 4ABP/Bz and 4ABP/(AcBz+Bz) were ~1.4-fold higher in the dye workers than in the Bz workers, but the ratios were not
significantly different (Table I). There was a 4-fold variation in the mean ratios of seven factories, but small numbers of workers in some factories precluded detailed analysis (data not shown). This suggests environmental contamination with 4ABP, although individual variation for the release of 4ABP from Bz and Bz dye is unknown. On the other hand, there were very strong correlations between adducts of 4ABP, Bz and AcBz among all exposed workers in the seven factories combined (Table II, Figure 1). This provides some support for endogenous release of 4ABP from Bz, as it is less likely that contamination of starting materials by 4ABP, Bz and AcBz products would have existed to a similar extent in the seven factories.

In rats given Bz (14) and 4ABP (22), 0.35 and 5% of the dose bind to Hb (Table III). In rats dosed with Bz, three adducts were found; AcBz, Bz and 4ABP bound to Hb at 0.274, 0.035 and 0.044% of the dose (14), so that the ratio of 4ABP/(AcBz + Bz) was equal to 0.14. In rats given the azo dye Direct Red 28, the same adducts that were found after Bz dosing were detected, and the ratio 4ABP/(AcBz + Bz) was 1.05 (14). In the Bz workers and the dye workers of our study, the mean ratios of 4ABP/(AcBz + Bz) were 3.03 and 4.26, respectively. In the Bz workers, this ratio was 20-fold higher than has been found in rats. In dye workers, who were mainly exposed to Direct Black 38 and not Direct Red 28, the adduct ratios are in the same order of magnitude as in rats. Thus, assuming that the same adduct ratios seen in rats are also present in humans, it is possible that 4ABP was present as a contaminant in the Bz factories, as it would seem that far more 4ABP Hb adducts were detected in the Bz workers than would be predicted on the basis of the animal data. This might be supported by the fact that AcBz, which is the major adduct of the two Bz adducts, did not correlate with 4ABP. Since in rats 4ABP binds ca. 20 times more than Bz (see above), theoretically 4ABP has to be present as a 5% contaminant to obtain the same Hb-adduct levels as AcBz. Taking into account all of the above information, it is likely that 4ABP Hb adducts derived from both endogenous release from Bz and contamination of the manufacturing materials, though definite conclusions cannot be made.

Cancer risk

We took advantage of this data set to estimate the theoretical cancer risk that would be experienced in this occupational setting. We detected large amounts of Hb adducts deriving from aniline, 4ABP and Bz. In order to estimate the cancer risk, the daily dose was estimated from the adduct levels. Since only 4ABP and Bz are human carcinogens and potent rodent carcinogens, the cancer risk calculations were performed with 4ABP and Bz only (Table III). The daily Bz dose of the worker was estimated from the Hb-adduct levels. It was assumed that the same percentage of the dose binds in rats and humans and that in humans the steady-state Hb-adduct level is 60 times larger than that after a single dose (18,27). For 4ABP, the percentage of the dose bound to Hb in rats is very similar to the percentage of the dose bound to Hb of smokers (27), although two additional cysteine groups are present in the alpha-chain of the Hb of rats (28) but not in humans or mice. The Hb-adduct levels for five arylamines is 2–28 times lower in mice than in rats (22). Therefore, the estimation of the daily dose from Hb adducts in rats could be underestimated. The daily dose for the average Bz+AcBz Hb-adduct level in Bz workers was estimated to be 81.3 (nmol/kg)/day. This would correspond to an air concentration of 109 μg Bz/m³ (ca. 0.014 ppm), assuming that a 70 kg worker breathes 9.6 m³ in an 8 h work-shift and that 100% Bz are absorbed. An excess lifetime cancer risk (ELCR) for exposure to Bz was estimated using the formula published by the USA regulatory agencies (29,30; http://risk.lsd.ornl.gov): ELCR = (cancer slope factor) × (human dose). Workers were not exposed 7 days per week and not all 52 weeks per year to the workplace contaminant. Since we deduced the external dose from the internal dose, days without exposure were included. The dose was corrected with the factor 40/70, since workers are not exposed for a lifetime of 70 years to the occupational pollutants, but for 40 years of their working life. This yields an ELCR > 100% (= 197%) for Bz. Therefore, all workers would develop bladder cancer according to this estimation over the course of a working lifetime. Such an instance has been reported in a small plant in England, where all 15 workers involved in distilling of 2-naphthylamine developed bladder cancer (31). Another way of estimating this human cancer risk is to use the human exposure rodent potency index (HERP index) (32), which is deduced from the daily dose administered to rodents that results in a 50% increased likelihood of tumor development (TD50) (33). The HERP index corresponds to a ratio of the daily human exposure and the rodent TD50 value expressed as percentage. The HERP calculated from the average Bz dose is 0.5%, which corresponds to
may be complicated by the potential presence of other arylamines.

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Conflict of Interest Statement: None declared.

References


