The Carcinogenicity of Methoxyl Derivatives of 4-Aminoazobenzene: Correlation between DNA Adducts and Genotoxicity

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To elucidate the cause of the difference in genotoxic activity between carcinogenic 3-methoxy-4-aminoazobenzene (3-MeO-AAB) and noncarcinogenic 2-methoxy-4-aminoazobenzene (2-MeO-AAB), we analyzed DNA adducts in the livers of rats exposed to either of these chemicals and studied the resulting biologic potential with the aid of in vitro modified M13 phage DNA. ³²P-Postlabeling analysis revealed that the carcinogen 3-MeO-AAB produced 20-fold higher amounts of adducts than did 2-MeO-AAB. Five adducts were formed in the 3-MeO-AAB case whereas only one adduct was apparent in 2-MeO-AAB-treated rats. Studies of in vitro DNA replication using N-hydroxy (N-OH)-aminoazo dye-modified M13 phage DNA as a template demonstrated inhibition by 3-MeO-AAB adducts to be substantially greater than in the 2-MeO-AAB adducts. The specificity of mutagenesis induced in M13mp9 phage DNA by these chemicals also was analyzed after transfection into SOS-induced Escherichia coli JM103, mutation frequencies being higher with N-OH-3-MeO-AAB than N-OH-2-MeO-AAB-modified DNA. The mutation spectra differed in each case. Our data suggest that the difference in hepatocarcinogenic activity between the two chemicals depends not only on qualitative and quantitative variation in adduct formation but also on conformation changes in modified DNA. — Environ Health Perspect 102(Suppl 6):191–194 (1994)

Key words: 3-methoxy-4-aminoazobenzene, 2-methoxy-4-aminoazobenzene, DNA adduct, ³²P-postlabeling, DNA synthesis, mutation M13 DNA

Introduction

Over several years, we have focused our attention on elucidation of the cause of the difference in genotoxic activity between 3-methoxy-4-aminoazobenzene (3-MeO-AAB) and 2-methoxy-4-aminoazobenzene (2-MeO-AAB) (1–9). The carcinogenic potencies of these aminoazo dyes are known to be influenced by the position of methoxy substituents on the aminoazobenzene molecule. 3-MeO-AAB is a hepatocarcinogen in the rat and a mutagen in Escherichia coli and Salmonella typhimurium, whereas 2-MeO-AAB, differing only in the position of the methoxy substituted on the benzene ring, is a noncarcinogen and nonmutagen (5–7).

This article concerns: a) ³²P-postlabeling analysis of DNA adducts in the liver after intraperitoneal (ip) administration of 3-MeO-AAB or 2-MeO-AAB, b) effects of DNA adducts on in vitro DNA synthesis, and c) mutations induced by aminoazo dye adducts in M13 viral DNA.

³²P-Postlabeling Analysis of Hepatic DNA Adducts in F344 Rats Treated with 3-MeO-AAB or 2-MeO-AAB

In vivo formation of hepatic DNA adducts after exposure to the aminoazo dyes was studied using ³²P-postlabeling analysis. Male F344 rats (5–6 weeks old) were given a single ip injection of 3-MeO-AAB or 2-MeO-AAB (50 mg/kg), and DNA was isolated from livers at 16, 24, 48, 72 hr and 1 week thereafter. Analysis of DNA adducts was carried out by the butanol extraction procedure of the ³²P-postlabeling assay (1,8).

Representative results are shown in Figure 1. DNA adducts formed by 3-MeO-AAB consisted of one major (no. 1) and four minor (nos. 2–5) spots, while 2-MeO-AAB gave only one spot (no. 1).

Figure 1. Autoradiographs of PEI-cellulose maps of DNA adducts in rat livers formed at 24 hr and 48 hr after treatment with 3-MeO-AAB (A) and 2-MeO-AAB (B), respectively. About 70 μCi and 140 μCi of ³²P-labeled digests for 3-MeO-AAB and 2-MeO-AAB, respectively, were chromatographed. Development was carried out in the first two dimensions as described (1,8), then in 3.5 M lithium formate, 8.5 M urea, pH 3.5 (D3), followed by 0.8 M LiCl, 0.5 M tris-HCl, 8.5 M urea, pH 8.0 (D4). Autoradiograms were developed for 24 hr at –80°C.
Maximal DNA binding of 3-MeO-AAB and 2-MeO-AAB was observed after approximately 24 and 48 hr, respectively, and thereafter decreased gradually (Table 1). After 7 days, 40% of the maximum adduct levels were still present in both cases. The maxima were 3.8 for 3-MeO-AAB and 0.16 for 2-MeO-AAB per 10^7 nucleotides. The carcinogen 3-MeO-AAB generated more than 20-fold higher amounts of adducts in the liver than did 2-MeO-AAB at all time points examined.

**Effects of 3-MeO-AAB- and 2-MeO-AAB-Adducts on in Vitro DNA Synthesis**

To determine the effects of DNA adducts caused by 3-MeO-AAB and by 2-MeO-AAB on the replication of DNA, we analyzed the reaction products of *E. coli* DNA polymerase I (pol I) action on aminoazo dye-modified M13mp10 DNA templates by DNA sequencing gel electrophoresis.

Single-stranded M13mp10 DNA was modified with N-hydroxy (N-OH) derivatives of 3-MeO-AAB or 2-MeO-AAB in the presence of seerl-AMP, whereby adducts have been proposed to be formed through electrophilic intermediate N^4^-O-seryl-oxynoamine derivatives (9,10). The extents of aminoazo dye adducts were calculated from spectrophotometric analyses (2,11).

Figure 2 shows representative sequencing gel-electrophoresis bands of primer elongation products. From the evidence of stronger arrested bands and shorter DNA products observed with the N-OH-3-MeO-AAB-modified template, a conclusion can be drawn in this case of more effective inhibition of DNA chain elongation. In clear contrast, the arrested bands with N-OH-2-MeO-AAB-modified template were much fainter and the DNA products generally were longer, suggesting that pol I was able to read through 2-MeO-AAB adducts.

Elongation was arrested extensively at one base prior to every 3-MeO-AAB guanine adduct and at -GGG-- sequences but not always at 2-MeO-AAB-guanine adducts, but it was blocked at adenines in -GAG-- sequences. The differences between 3-MeO-AAB and 2-MeO-AAB guanine adducts might be because of their chemical structures or surrounding altered structures of the DNA.

**Table 1. DNA adducts formed by 3-MeO-AAB and 2-MeO-AAB in rat livers in vivo.**

| Azo dye | Adduct numbera | Number of DNA adducts/10^9 nucleotides |
|---------|----------------|----------------------------------------|
|         |                | 16 hr | 24 hr | 48 hr | 72 hr | 7 d |
| 3-MeO-AAB | 1      | 15.0 ± 5.0⁷ | 26.0 ± 1.2 | 20.2 ± 2.4 | 17.3 ± 0.5 | 9.3 ± 1.8 |
| 3-MeO-AAB | 2      | 1.5 ± 0.1   | 2.5 ± 0.9  | 2.9 ± 0.5  | 1.4 ± 0.1  | 1.0 ± 0.1  |
| 3-MeO-AAB | 3      | 1.6 ± 0.1   | 2.4 ± 0.6  | 2.1 ± 0.9  | 1.9 ± 0.5  | 1.4 ± 0.4  |
| 3-MeO-AAB | 4      | 1.0 ± 0.2   | 2.5 ± 1.3  | 1.4 ± 0.5  | 1.1 ± 0.3  | 0.9 ± 0.4  |
| 3-MeO-AAB | 5      | 1.9 ± 0.3   | 2.6 ± 1.0  | 1.9 ± 0.5  | 1.6 ± 0.4  | 1.3 ± 0.4  |
| Total    |        | 21.0 ± 5.6  | 36.0 ± 5.0 | 27.6 ± 0.1 | 23.3 ± 0.9 | 13.9 ± 0.5 |

| 2-MeO-AAB |                | 16 hr | 24 hr | 48 hr | 72 hr | 7 d |
|-----------|----------------|------|------|------|------|------|
| 2-MeO-AAB | 1      | 0.3 ± 0.01 | 0.6 ± 0.1 | 1.6 ± 0.3 | 1.3 ± 0.2 | 0.6 ± 0.03 |

aAdduct numbers correspond to those of spots in Figure 1. Radioactivity of each adduct and total nucleotide spot was determined by Cerenkov counting. bMean ± SE of data for two separate tissues. DNA adduct levels in each tissue were calculated from duplicate samples.

**Figure 2. Analysis of products synthesized by pol I using M13 DNA template containing DNA lesions.** One pmoles of each template DNA containing 8 to 10 adducts per DNA molecule was annealed with 1 pmoles of the primer, and elongation by pol I was carried out as described previously (2). Lane 1, N-hydroxy-3-methoxy-4-aminobenzene; lane 2, N-hydroxy-2-methoxy-4-aminobenzene; lane 3, 4-hydroxyaminomethylquinoline-1-oxide; lane 4, unmodified DNA; lanes 5 to 8, as for lanes 1 to 4, respectively, except that a chase reaction for 20 min was carried out. The lanes T, A, C, and G represent standard Sanger dideoxyribonucleotide sequence ladders.
in agreement with our previous finding that these aminoazo dyes specifically form adducts with guanine bases (11).

Discussion

The aim of this study was to elucidate differences in genotoxic activity between 3-MeO-AAB and 2-MeO-AAB. 32P-Postlabeling analysis of DNA adducts from the liver of rats exposed to these chemicals revealed that the carcinogen 3-MeO-AAB produced 20-fold higher amounts of adduct than the noncarcinogen 2-MeO-AAB. Moreover, five spots were detected on TLC sheets from 3-MeO-AAB-treated rats, whereas only one adduct was found in 2-MeO-AAB-treated rats. Essentially similar results were obtained with DNA of E. coli uvrA strains treated with N-OH-3-MeO-AAB or N-OH-2-MeO-AAB (3).

Although the adducts formed by 3-MeO-AAB and 2-MeO-AAB have not yet been fully characterized, C8-substituted deoxyguanosine (spot no. 1) may well be a major product. Minor spots (no. 2–5) in 3-MeO-AAB-modified DNA may be deoxyguanosin N4-yl-substituted and deoxyadenosin N6-yl-substituted 4-aminoazobenzene derivatives (13,14). In this context, it is of particular importance that the kind of DNA damage—the nature of the adduct itself or the conformational changes in DNA—which is responsible for the biologic activity of 3-MeO-AAB be clarified.

We therefore compared the biologic potential of M13 phage DNA in vitro modified by these chemicals. As a result, DNA replication by pol I was found to be blocked at or one base prior to guanine bases in the templates, 3-MeO-AAB adducts having a significantly greater inhibitory effect on DNA synthesis than 2-MeO-AAB adducts. The arrested pattern also differed in each case.

While 3-MeO-AAB adducts induced mutations more frequently than did 2-MeO-AAB adducts, they were both mainly derived from guanine base. However, 3-MeO-AAB adducts induced a higher percentage of frameshift mutations as well as base substitutions, suggesting a possibly greater alteration in DNA conformation. In contrast, 2-MeO-AAB adducts induced mostly base substitutions.

Our data thus suggest that the difference in hepatocarcinogenic activity between 3-MeO-AAB and 2-MeO-AAB depends not only on qualitative and quantitative variations in adduct formation, but also on conformational changes in DNA modified by these chemicals.

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