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Lack of Binding of Methapyrilene and Similar Antihistamines to Rat Liver DNA Examined by ^{32}P Postlabeling[1]

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ABSTRACT

The nonmutagenic carcinogen methapyrilene, together with several noncarcinogenic analogues, was administered to rats p.o. for as long as 4 wk at concentrations of 0.1%. DNA was isolated from the liver and other organs and hydrolyzed, and the identification of covalent adducts was made using the ^{32}P postlabeling method of Randerath. Some modified procedures were also used to deal with the possibility of very mobile adducts being formed from these hydrophilic amines. Although the rats had received as much as 2 g of amine per kg of body weight, no evidence of formation of DNA adducts in liver or other organs was seen; the level of detection was between 1 in 10^8 and 1 in 10^9 nucleotides. Adduct formation from much lower doses of the mutagenic food pyrolysis product 2-amino-3-methylimidaz(4, 5f)quinoline was detectable at a level of 1 in 10^6 nucleotides in parallel analyses. These results add to the evidence that carcinogenesis by methapyrilene is through an indirect or nonmutagenic mechanism.

INTRODUCTION

Many carcinogens are not mutagenic in the usual bacterial or mammalian cell mutagenesis assays. Among the apparently nonmutagenic carcinogens are some which induce liver tumors in rats, yet are not activated to mutagens by rat liver microsomal fractions. Before these anomalous carcinogens can be classified as "nongenotoxic," however, it seems necessary to be sure that the reason for their inactivity in assays such as the "Ames" *Salmonella* reversion assay is not the insensitivity of those assays to small mutational effects of the compounds. The recently introduced postlabeling method for detecting DNA adducts of large, usually aromatic, carcinogens (1) affords an opportunity for observing interactions at very low levels and avoiding the possibility of artifacts, which use of very high specific activity-labeled carcinogens comprise, when specific activities in the DNA are below those at which impurities in the starting material are likely to be present.

One recently discovered carcinogen to which considerable numbers of people have been exposed is the antihistamine methapyrilene, which does not resemble in chemical structure any previously known carcinogen. Methapyrilene induces liver tumors (hepatocellular carcinomas and cholangiocellular carcinomas) in rats following chronic p.o. administration (2). However, it did not induce tumors in Syrian hamsters or in Guinea pigs (3). There are very few clues to the mechanism of carcinogenesis by methapyrilene, since it has been nonmutagenic to bacteria (4) with rat liver activation and nonmutagenic to *Drosophila*,[4] does not induce sister-chromatid exchange (5), and has failed to transform mammalian cells in culture under conditions in which many complex carcinogens are transforming (6). Methapyrilene is not highly toxic, and almost its only known biological action is to induce proliferation of mitochondria in rat liver (7), but not in the liver of hamsters or Guinea pigs (8). Methapyrilene has other effects on rat liver, which are difficult to relate to carcinogenesis, for example, increasing lipid peroxidation (9); this property is shown equally, however, by the noncarcinogenic analogues thenyldiamine and methafurylene (10). None of the chemically similar, but noncarcinogenic (11) analogues of methapyrilene induces mitochondrial proliferation in rat liver (8). These analogues, methaphenilene, thenyldiamine, methafurylene, and chlorothen, each differ in only one structural particular from the carcinogen methapyrilene, which makes their noncarcinogenicity an interesting puzzle. The failure of labeled methapyrilene to localize in the nuclei of liver cells of rats, although the mitochondria of rat liver cells are extensively radiolabeled (7), confirms earlier chemical findings that DNA of liver cell nuclei was not significantly radiolabeled, following administration of as much as 1 mCi of radiolabeled methapyrilene to rats, whereas soluble liver protein was extensively labeled (12).

The unusual effectiveness of methapyrilene in inducing liver tumors in rats, comparable with several genotoxic carcinogens in potency, raised the question whether previous studies of its interaction with DNA *in vivo* might have been too crude or too insensitive. Many cyclic nitrosamines, which are genotoxic carcinogens, have failed to yield measurable, identifiable DNA adducts using radiolabeled carcinogen as the probe (13). The recent introduction by Randerath of a very sensitive method for detecting formation of DNA adducts with nonradiolabeled carcinogens (1, 14) afforded the opportunity to investigate binding of methapyrilene to rat liver DNA and to compare that binding with analogues of methapyrilene which do not induce tumors in rat liver.

MATERIALS AND METHODS

Animal Treatments. Methapyrilene hydrochloride was from Sigma Chemical Co., St. Louis, MO. Methafurylene, methaphenilene, and chlorothen were synthesized as described previously (11). The compounds were dissolved in water at a concentration of 1 g per liter by addition of a few drops of 10 N HCl. The four 0.1% solutions were offered to two cages of three 8-wk-old male Fischer rats, at the rate of 60 ml per cage per day. Sucrose was added, 20 to 30 g per liter, to the solutions to make them more palatable. A group of six rats in two cages was given only water to drink and served as controls. Solutions of methapyrilene, methaphenilene, and methafurylene were mostly consumed—to the extent of 60 to 70 ml per rat in the first week. The rats drank the chlorothen solution only when it was diluted to 0.5 g per liter at the end of the first week.

At the end of the first week's treatment, three rats treated with methapyrilene, methafurylene, and methaphenilene were killed. The

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[1] Since this manuscript was prepared, D. A. Casciano, J. G. Shaddock, and G. Talaska (Mutat. Res., *208:* 129–135, 1988) reported a similar result, namely, 24 h following i.p. injection of 75 to 250 mg/kg of body weight of methapyrilene hydrochloride, they failed to find DNA adducts in rat liver by ^{32}P postlabeling.

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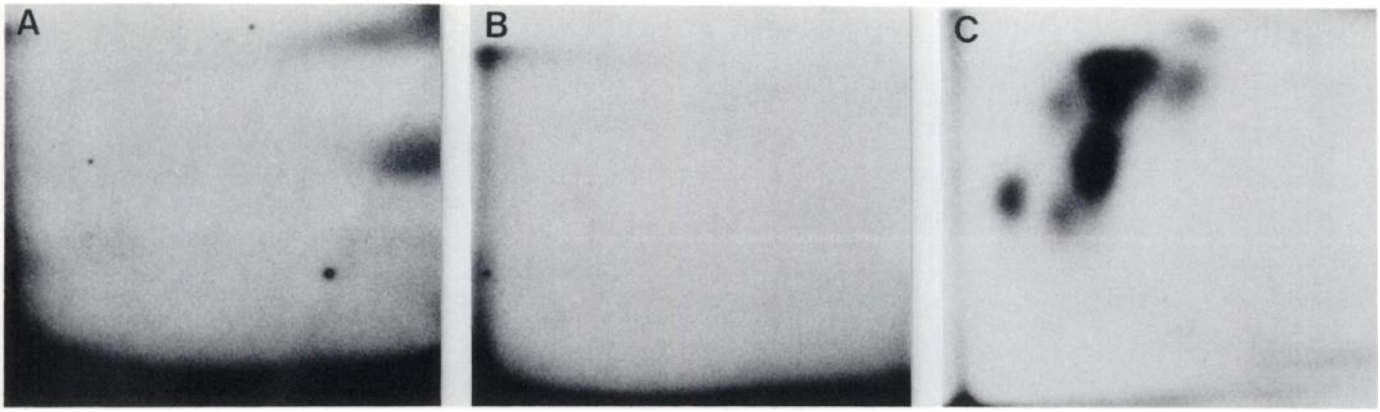

Fig. 1. Autoradiographs of chromatograms of ^{32}P-labeled nucleotides from liver DNA of rats: *A*, untreated control; *B*, 0.1% methapyrilene in the diet for 1 mo; *C*, 50 mg/kg of IQ 24 h previously.

remaining group on each treatment was continued for a second week, after which the second cage of rats was sacrificed. The chlorothen-treated rats were sacrificed after 2 and 3 wk of treatment. At that time, chlorothen-treated rats weighed only 94 g, compared with 135 to 150 g for both the controls and the rats treated with the other three antihistamines. The calculated average intake of the compounds during 2 wk of treatment was 80 mg of chlorothen and 120 to 140 mg of methapyrilene, methafurylene, or methaphenilene, or approximately 1 g per kg of body weight of the last three compounds.

DNA Analysis. After sacrifice, the liver, kidneys, and lungs of each rat were dissected and instantly frozen on a metal block at liquid nitrogen temperature. From each organ of one of the rats at the 1-wk time point in each treatment DNA was isolated according to a standard procedure (15). One DNA liver sample from each of the treatments and a control was analyzed using the postlabeling procedure (1, 14), as described for such carcinogens as aromatic amines. DNA (5 μg) was digested according to Gupta *et al.* (1), and ^{32}P postlabeling was carried out under adduct intensification conditions (14).

Because methapyrilene and the other antihistamines are not hydrophobic like the aromatic amines, but are very hydrophilic, the failure to detect adducts using the standard postlabeling method might have been fortuitous. Therefore, a modification of the procedure for hydrophilic compounds, using a different substrate for chromatography (16), was used. To retain adducts by hydrophilic compounds, labeled materials were spotted on a C_{18}-reversed-phase thin-layer chromatography plate (KC18 plate; Whatman) and developed in 0.4 M ammonium formate at 4°C for 15 h (16). Adducts at the origins were transferred to PEI-cellulose sheets (Polygram Cell 300 PEI; Marchery-Nagel, Düren, West Germany) (17) and developed with buffer as shown in Table 1. Final development was with 1.7 M sodium phosphate (pH 6.0) with 1.5-cm paper wick from left to right.

A further experiment was conducted in which methapyrilene hydrochloride mixed in the diet at 0.1% was fed to rats for 4 wk, a treatment which led to 100% incidence of liver tumors when continued for a year (2). The rats were killed; DNA was isolated from liver, kidney, lung, colon, and stomach; and adducts were separated and identified by ^{32}P postlabeling, by methods described above.

As a positive control, liver DNA from rats that had been fed the carcinogenic polynuclear amine IQ,[5] found in broiled fish (18), was used. Rats were given 50 mg of IQ/kg of body weight by gavage and sacrificed 24 h later. The presence of adducts which could be easily identified in this DNA is well established, and the amount of DNA used in the control study was much smaller than that of the DNA from the liver of antihistamine-treated rats. After isolation of DNA from the liver, the IQ adducts were separated by chromatography as above, using the development schedule listed in Table 2.

Autoradiography with intensifying screen (Dupont Lightning Plus) was used to locate spots containing ^{32}P-labeled adducts (Fig. 1).

Table 1 *Methodology of adduct development with buffer*

Direction 1 (from bottom to top)	Direction 2 (from left to right)
System 1: 2.3 M lithium formate 4.3 M urea (pH 3.5)	0.5 M lithium chloride 0.25 M Tris-HCl 4.3 M urea (pH 8.0)

Table 2 *IQ adduct separation by chromatography*

Direction 1 (from bottom to top)	Direction 2 (from left to right)
2.7 M lithium formate 5.1 M urea (pH 3.5)	0.8 M lithium chloride 0.4 M Tris-HCl 6.8 M urea (pH 8.0)

RESULTS AND DISCUSSION

Autoradiography of the chromatograms of the liver DNA preparations from the 1-wk treatments with the antihistamines contained no spots which would reflect the presence of nucleotides containing bulky adducts. The sensitivity of the procedure was sufficient to detect adducts present at a level of between 1 in 10^8 and 1 in 10^9 nucleotides. However, the chromatographic procedure used could lead to loss of adducts of hydrophilic compounds, such as the very polar amines represented by the antihistamines, to deoxynucleotides.

The modified procedure, designed to detect adducts of less hydrophobic compounds, showed the presence of adducts in the liver DNA of rats treated with IQ, which were easily seen and present at a level of approximately 1 in 10^6 nucleotides (Fig. 1). In contrast, there were no adducts discernible in the liver DNA of rats treated with methapyrilene, methafurylene, methaphenilene, or chlorothen, at the end of the second week of treatment. The sensitivity of the assay was higher than 1 in 10^8 nucleotides, so that absence of DNA adducts at this level is indicated. Because there was no indication of adducts in the liver, a target organ, it was not considered worthwhile to examine the DNA from the kidney or lung of rats fed the antihistamines.

The results of the examination of liver DNA from the rats fed methapyrilene hydrochloride for 4 wk are shown in Fig. 1*B*, together with the autoradiograph derived from the DNA of untreated rat liver (Fig. 1*A*), and from IQ-treated rat liver (Fig. 1*C*). The DNA from the other organs of the methapyrilene-treated rats gave equally negative autoradiographs. These are not shown.

There is no indication from these results that methapyrilene forms adducts with DNA. Althaus *et al.* (19) reported damage to cellular DNA *in vitro* by high, toxic concentrations of methapyrilene. The relevance of the report of Lampe and Kammerer

[5] The abbreviation used is: IQ, 2-amino-3-methylimidaz(4,5-f)quinoline.

(17) that methapyrilene binds to calf thymus DNA *in vitro* is not clear. There must be alternative ways of inducing tumors rather than by direct mutation of DNA through adduct modification. The peroxisome proliferators clofibrate and ciprofibrate also failed to form detectable DNA adducts (at a level of 1 in 10^{10}) in rat liver (20), although they induce liver tumors in rats, as does the mitochondrial proliferator methapyrilene.

There is the philosophic question whether adducts of methapyrilene in liver DNA could be present at still lower levels and what the effect of them might be. Debating this is not a worthwhile pursuit, since there is an equal chance that there would be found lower levels of adducts of the noncarcinogenic antihistamines as of the carcinogenic methapyrilene. Until shown otherwise, it can be assumed that methapyrilene, although a rat liver carcinogen, does not form adducts with the DNA of rat liver *in vivo*. This is in accordance with the failure of methapyrilene to show genotoxicity in the other assays used to demonstrate it, with the exception of a weak response in the mouse lymphoma assay (21). Methapyrilene and some other carcinogens remain intriguing exceptions to the general hypothesis of mechanisms of carcinogenic activity.

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