Inability of Methapyrilene to Induce Sister Chromatid Exchanges in Vitro and in Vivo

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ABSTRACT

The induction of sister chromatid exchanges (SCE) by the hepatocarcinogen methapyrilene hydrochloride was investigated using appropriate in vitro and in vivo mammalian cell systems. Methapyrilene, even at the maximum tolerated dose, did not induce SCE in Chinese hamster ovary cells (CHO) or when CHO cells or hamster lung fibroblasts, V-79, were cocultivated with early cultures of rat liver epithelial cells, which are known to metabolize different classes of chemical carcinogens to active forms. Moreover, a hybrid clone of cells (formed by fusion of CHO cells with rat liver epithelial cells), which is highly sensitive to SCE formation by a number of xenobiotics, failed to produce SCE after treatment with methapyrilene. Experiments in vivo with bone marrow cells and in vitro with CHO cells cocultivated with primary hepatocytes from rats also confirmed the inability of methapyrilene to induce SCE in the indicator cells. Since aflatoxin B1 induced SCE in the in vitro and in vivo models, it may be concluded that methapyrilene does not induce SCE at a concentration which is not cytotoxic to the indicator cells in the different systems described. Autoradiographic studies in cultured rat liver cells with tritiated methapyrilene showed that the label was localized in the cytoplasm but not in the interphase nuclei or in the metaphase chromosomes, indicating a lack of interaction of methapyrilene with the nuclear macromolecules of the putative target cells for methapyrilene.

INTRODUCTION

The antihistaminic methapyrilene is reported to be a potent hepatocarcinogen (16) which does not significantly react with rat liver DNA, but soluble proteins isolated from liver contain considerable bound material (15). It is also known that SCE in different indicator cells types are induced by carcinogenic chemicals which interact with DNA (3, 21). Despite its nonmutagenic property in the Salmonella mutagenicity assay of Ames (2) and its inability to induce unscheduled DNA synthesis in freshly isolated rat liver cells (23), methapyrilene or its metabolites might induce SCE indirectly (e.g., through reaction with nuclear proteins or through the production of free radicals). Moreover, Althaus et al. (1) recently published data which suggest that unscheduled DNA synthesis is indeed induced by methapyrilene in primary cultures of freshly isolated rat liver cells. They also found that this drug may be a direct-acting carcinogen. We investigated the SCE-inducing properties of methapyrilene, using appropriate in vitro and in vivo mammalian cell systems. SCE were not induced in any of the systems and radioactivity from [3H]methapyrilene was localized primarily in the cytoplasm of the early cultures of liver epithelial cells.

MATERIALS AND METHODS

Test Chemicals. Methapyrilene hydrochloride (Lot 48C-0022) was purchased from Sigma Chemical Co. (St. Louis, Mo.). [3H]Methapyrilene (specific activity, 1.3 Ci/mmol) was obtained from a sample prepared as described elsewhere (15). [3H]DMBA (specific activity, 37 Ci/mmol), purchased from the Radiochemical Center, Amersham/Searle Corp. (Arlington Heights, Ill.), was chromatographically purified (5) before use. Mitomycin C and aflatoxin B1 were obtained from Calbiochem (San Diego, Calif.) and Sigma, respectively.

Cell Lines. Chinese hamster ovary cell line CHO was purchased from the American Type Culture Collection, Rockville, Md. (CCl 61CHO-K1). Chinese hamster lung fibroblasts, V-79, were obtained from Dr. B. Myhr, Litton Bionetics, Kensington, Md. Rat liver epithelial cell lines LNRL and FNRL were prepared in our laboratory from Lewis and Fischer strains of rats, respectively, by previously described methods (8) and were used between the 10th and 15th passages. The hybrid clone 3.1.9 derived from fusion of LNRL and CHO cells (10) was also used in these studies.

Culture Methods. All the cell lines were cultured routinely in Ham's F-10 medium (6) supplemented with 10% fetal bovine serum (K. C. Biologicals, Inc., Lenexa, Kans.). The cells were grown on plastic Petri dishes (Falcon Plastics, Inc., Oxnard, Calif.) and incubated at 37° in humidity cabinets with a gas phase of 5% CO2 in air.

Viability of Cells with Methapyrilene. Single-cell suspensions of the cell line were plated at a cell density of 20 cells/sq cm (500 cells in 5 ml of medium in 50-mm dishes). After 1 day, the medium was removed and an equal volume of either the control medium or media with varying concentrations of the test chemicals was added to the cultures. The cells were then mixed at a ratio of 1:1 and plated as above. After 48 hr, the culture medium was removed, and an equal volume of either the control medium or media with a different concentration of methapyrilene was added to the cultures. The cultures were refed on the 6th day with appropriate media, and on the 11th day cells were fixed in methanol and stained with Giemsa. The colonies were counted with an automatic colony counter (a minimum of 3 dishes/dose).

SCE Induction in Vitro. Single-cell suspensions trypanized from monolayers were plated at a density of 1 × 105 cells/dish in 5 ml of culture medium. In some experiments, the CHO and LNRL or freshly isolated rat liver cells, or V-79 and FNRL cells, were mixed at a ratio of 1:1 and plated as above. After 48 hr, the culture medium was removed, and an equal volume of medium containing BrdUrd (10 μg/ml; Sigma) and varying concentrations of the test chemicals was added to the culture. Control cultures received medium containing BrdUrd. The cultures were kept in complete darkness to minimize the induction of SCE in vivo. After an additional 24 hr of incubation, during which the cells went through 2 rounds of DNA replication, Colcemid (0.02 μg/ml; Grand Island Biological Co., Grand Island, N. Y.) were added to arrest the cells in mitosis, and 2 hr later the cells were harvested using 0.05% trypsin. The cells were suspended in a hypotonic solution (0.075 M KCl) for 20 to 30 min and then fixed in methanol:acetic acid (3:1) for a minimum of 30 min. After 3 changes of fixative, the cells were spread on microscope slides and air dried. Some of the slides were stained for 15 min in 5% Giemsa stain, followed by dipping in 1% aqueous aqueous methylene blue (0.05%), drying, and mounting with a coverslip.
R66 diluted in a pH 6.8 buffer, both obtained from G. T. Gurr, Hopkin and Williams, Chadwell Heath, Essex, United Kingdom. Other slides were stained by a modified fluorochrome-plus-Giemsa technique (22, 25).

**SCE Induction in Vivo.** Male Fischer rats (6 weeks old; average weight, 125 g) were given i.p. injections of BrdUrd (30 μg/g body weight) every 30 min for a 10-hr period. Fifteen min after the last injection, the test chemicals were administered by gavage. Colchicine was injected i.p. (2 μg/g body weight) 11 hr after treatment with the carcinogen, and the rats were killed 1 hr later. The marrow cells were isolated by aspirating the hypotonic solution (0.075 M KCl) through the femur bones with a 21-gauge needle. The cells were processed as described above.

Metaphase plates of the various cells containing differentially stained chromosomes were examined with an oil immersion objective using a Zeiss Universal microscope. The SCE present in 50 metaphase plates selected at random were counted for each concentration of the test compounds in all the cell systems. Every experiment had its own control SCE, and the data were analyzed statistically.

**Autoradiographic Studies.** The rat liver cell line FNRL was plated at a concentration 2 × 10^6 cells/ml in 5 ml of culture medium either in 60-mm Petri dishes or on glass coverslips. The culture medium was changed on the second day after plating, and an equal volume of medium containing either [3H]methapyrilene (4.3 μCi, 1 μg/ml) or [3H]DMBA (3.7 μCi; 0.025 μg/ml) was added, and the cells were incubated for 24 hr. The coverslips were rinsed three times with Dulbecco’s phosphate-buffered saline (Grand Island Biological Co.) containing an excess of the appropriate unlabeled carcinogens, methapyrilene or DMBA. The cells were then fixed with methanol:acetic acid (3:1); and the coverslips were washed repeatedly with water to remove the noncovalently bound methapyrilene and/or its metabolites or, in the case of DMBA samples, additionally washed with benzene to remove unbound DMBA and air dried. The coverslips were attached to glass slides with the cells facing upwards, using Permount (Fisher Scientific Co., Fair Lawn, N. J.). The cells grown on the dishes were treated with Colcemid for the last 3 hr of incubation, and chromosomes were prepared as described above. The coverslips and the chromosome preparations were dipped in N TB 2 nuclear track emulsion (Kodak, Arlington, Va.), dried and exposed for 4 to 8 weeks, and then developed with Kodak D-19 developer. Controls (cells not incubated with the labeled compounds) for the intact cells and the chromosome preparations were also used for autoradiography. The slides were lightly counterstained with Giemsa and analyzed with a Zeiss Universal microscope.

**RESULTS**

The effect of methapyrilene on cell viability was determined in the rat liver cell line FNRL as well as in the indicator cell lines CHO and a hybrid clone derived from fusion of rat liver cells with CHO cells. Treatment of the cells for 10 days with methapyrilene did not produce toxicity up to a dose of 10 μg/ml (Table 1). At a dose of 50 μg/ml, the relative plating lines. Although the inhibition of plating efficiency in CHO cells was also significant, it was lower than that seen in FNRL and the hybrid cell line. All the cell lines were very sensitive to a 100-μg/ml dose of methapyrilene (Table 1).

Methapyrilene did not show any induction of SCE in CHO cells cultured alone at any of the concentrations used (Table 2). An upper limit of 80 μg/ml was set for methapyrilene, because this concentration inhibited about 50% of the cells from entering into the second mitosis in the presence of BrdUrd. No induction of SCE was seen in those cells which did undergo 2 rounds of DNA synthesis and which showed clear differentiation of sister chromatids. Since the direct-acting agent mitomycin C (21) produced a 7-fold increase in SCE over the control, it seemed likely that methapyrilene may need metabolic activation before SCE can be induced. Since the commonly used indicator cell types (CHO; V-79) have little or no capacity to metabolically activate chemicals to derivatives that interact with cellular macromolecules, we have used appropriate activating cell systems. In experiments in which the indicator cells are cocultivated with the rat liver cells from the early passages of the cell lines known to metabolize a variety of chemical carcinogens (11, 25), or where the hybrid cells (10) which are very sensitive to SCE induction by directly or indirectly acting mutagenic carcinogens are used as the indicator cells, methapyrilene showed no effect on SCE production (Table 2). Under the same experimental conditions, the appropriate positive agent, aflatoxin B₁, did induce SCE. Early cultures of the nonmalignant liver cells could not be used as indicator cells for assessing SCE, since they do not undergo 2 rounds of DNA synthesis in the presence of BrdUrd (25), which is a prerequisite for visualizing sister chromatid differentiation. We have therefore used primary cultures of rat liver cells for cocultivation with CHO cells, but SCE were not induced in the indicator cells by methapyrilene (Table 2). Moreover, methapyrilene was ineffective even in bone marrow cells in rats gavaged with the chemical, whereas aflatoxin B₁ showed a significant induction of SCE in this system (Table 2). No increased frequencies of chromosome breaks over control values were seen in any of the cell systems used. However, with the higher dose used (80 μg/g body weight), there was a reduction in the percentage of cells showing differentiation of sister chromatids.

Since a high level of binding of [3H]methapyrilene to proteins isolated from liver was reported (15), we have used autoradiographic techniques to localize the covalently bound radioactivity in one of the rat liver cell lines (FNRL) used for the cocultivation experiments. The results are shown in Figs. 1 to 3. In the intact rat liver cell line FNRL, grown on coverslips and treated with [3H]methapyrilene, the label was found mainly in...
Table 2

Effect of methapyrilene on SCE induction in different indicator cells and experimental systems

<table>
<thead>
<tr>
<th>Indicator cells</th>
<th>Test compound</th>
<th>Positive compound</th>
<th>Dose of compound (µg/ml)</th>
<th>SCE(^a)</th>
<th>Ratio of treated vs. control</th>
<th>(p^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHO cultured alone</td>
<td>Methapyrilene</td>
<td>0</td>
<td>0.58 ± 0.03</td>
<td>0.36 - 0.80</td>
<td>NS(^c)</td>
<td>0.91</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>0.53 ± 0.04</td>
<td>0.36 - 0.77</td>
<td>NS</td>
<td>0.91</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20</td>
<td>0.50 ± 0.03</td>
<td>0.35 - 0.83</td>
<td>NS</td>
<td>0.87</td>
</tr>
<tr>
<td></td>
<td></td>
<td>40</td>
<td>0.58 ± 0.02</td>
<td>0.26 - 0.75</td>
<td>NS</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>80</td>
<td>0.55 ± 0.03</td>
<td>0.42 - 0.72</td>
<td>NS</td>
<td>0.95</td>
</tr>
<tr>
<td></td>
<td>Mitomycin C</td>
<td>0</td>
<td>0.58 ± 0.03</td>
<td>0.36 - 0.80</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.003</td>
<td>4.08 ± 0.20</td>
<td>2.78 - 4.73</td>
<td>6.95 &lt;0.001</td>
<td></td>
</tr>
<tr>
<td>CHO + LNRL mixed culture</td>
<td>Methapyrilene</td>
<td>0</td>
<td>0.57 ± 0.04</td>
<td>0.21 - 0.89</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>0.51 ± 0.02</td>
<td>0.40 - 0.60</td>
<td>NS</td>
<td>0.90</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20</td>
<td>0.58 ± 0.03</td>
<td>0.37 - 0.73</td>
<td>NS</td>
<td>1.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>80</td>
<td>0.52 ± 0.03</td>
<td>0.41 - 0.68</td>
<td>NS</td>
<td>0.91</td>
</tr>
<tr>
<td>V79 + FNRL mixed culture</td>
<td>Methapyrilene</td>
<td>0</td>
<td>0.54 ± 0.02</td>
<td>0.33 - 0.95</td>
<td>NS</td>
<td>0.11</td>
</tr>
<tr>
<td></td>
<td></td>
<td>40</td>
<td>0.55 ± 0.02</td>
<td>0.33 - 0.94</td>
<td>NS</td>
<td>0.11</td>
</tr>
<tr>
<td>CHO + LNRL mixed culture</td>
<td>Aflatoxin B(_1)</td>
<td>0</td>
<td>0.57 ± 0.04</td>
<td>0.21 - 0.89</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>1.44 ± 0.05</td>
<td>1.15 - 1.82</td>
<td>2.53 &lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Hybrid clone 3.1.9(^d)</td>
<td>Methapyrilene</td>
<td>0</td>
<td>0.47 ± 0.01</td>
<td>0.26 - 0.88</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>40</td>
<td>0.46 ± 0.01</td>
<td>0.20 - 0.71</td>
<td>NS</td>
<td>0.97</td>
</tr>
<tr>
<td></td>
<td>Aflatoxin B(_1)</td>
<td>0</td>
<td>0.47 ± 0.01</td>
<td>0.26 - 0.88</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>0.96 ± 0.03</td>
<td>0.50 - 1.47</td>
<td>2.02 &lt;0.001</td>
<td></td>
</tr>
<tr>
<td>CHO + primary hepatocytes mixed culture</td>
<td>Methapyrilene</td>
<td>0</td>
<td>0.33 ± 0.02</td>
<td>0.20 - 0.55</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>40</td>
<td>0.30 ± 0.02</td>
<td>0.16 - 0.45</td>
<td>NS</td>
<td>0.91</td>
</tr>
<tr>
<td></td>
<td>Aflatoxin B(_1)</td>
<td>0</td>
<td>0.33 ± 0.02</td>
<td>0.20 - 0.55</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>0.89 ± 0.04</td>
<td>0.58 - 1.26</td>
<td>2.70 &lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Bone marrow cells in vivo</td>
<td>Methapyrilene</td>
<td>0</td>
<td>2.50 ± 0.25</td>
<td>0 - 6</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>40(^e)</td>
<td>2.20 ± 0.24</td>
<td>0 - 5</td>
<td>0.88 NS</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Aflatoxin B(_1)</td>
<td>0</td>
<td>2.50 ± 0.25</td>
<td>0 - 6</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>6.55 ± 0.36</td>
<td>3 - 11</td>
<td>2.62 &lt;0.001</td>
<td></td>
</tr>
</tbody>
</table>

\(a\) Results are expressed as SCE/cell for the freshly isolated bone marrow cells in which there was no variation in the diploid number of chromosomes within the samples examined. In the case of cultured cells and especially in the hybrid clone, there was considerable variation in the number of chromosomes within the samples; hence, the results are expressed as SCE/chromosome for these samples.

\(b\) Probability associated with tests of control versus treated values using Student’s t test.

\(c\) NS, not significant; \(p > 0.25\) in these samples.

\(d\) See Ref. 10 for nomenclature of the hybrid clone of rat liver and hamster ovary cells.

\(e\) µg/g body weight.

The cytoplasm (Fig. 1). The nucleus contained hardly any grains over that of the background. Similarly, in the chromosome preparations, the metaphase chromosomes (Fig. 2A) as well as the interphase nuclei (Fig. 2B) did not show grains over the control. In contrast, in \(^{[3H]}\)D MBA-treated cells, both the metaphase chromosomes (Fig. 3A) and the interphase nuclei (Fig. 3B) contained the grains.

**DISCUSSION**

It is becoming increasingly clear that SCE in different indicator cell types (3, 21) are induced by carcinogenic chemicals which interact with nuclear DNA. However, certain chemicals which do not interact directly with DNA (e.g., tumor-promoting agents) are also known to enhance SCE production (13, 20, 24, 26). Therefore, even though methapyrilene gave negative results in mutagenicity tests (2) and gave conflicting results as to the ability to induce unscheduled DNA synthesis in freshly isolated liver cells (1, 23), we investigated the effects of various doses of this hepatocarcinogen on the viability of and SCE production in the various cell culture systems.

Although methapyrilene appears to be more cytotoxic to rat liver cells and to a rat liver-CHO hybrid cell clone than to CHO cells (Table 1), this result does not prove that metabolism of methapyrilene is carried out by any of the cells or that metabolism is required for the induction of cytoxicity. The latter could well be the result of direct action of this compound with any of the cellular organelles or macromolecules which may or may not include the nucleus and/or nuclear DNA. Cytotoxicity was observed by others (23) when 500- to 1000-nmol/ml (150- to 300-µg/ml) doses of the drug were added to freshly isolated rat liver cells. The colony assay reported here gives a precise end point for cell viability, and a concentration of 50 µg/ml is shown to produce a significant inhibition of cell viability.

Methapyrilene did not induce or enhance SCE over the control value in any of the in vitro systems whether the indicator cells were cultured alone or cocultivated with early cultures of rat liver epithelial cells (Table 2). These results suggest that (a) methapyrilene may not be a direct DNA-damaging agent, (b) the cultured liver cells and the liver cell/CHO hybrid cell clones may be incapable of producing reactive metabolites from meth-
The lack of SCE induction by methapyrilene seen in this study may be attributed to the inability of this drug to react with the nuclear macromolecules; therefore, this hepatocarcinogen does not appear to manifest one of the characteristics of genotoxic carcinogens such as aflatoxin B₁. The mechanism by which methapyrilene produces hepatocellular carcinomas is not clear at present and merits further research.

ACKNOWLEDGMENTS

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REFERENCES


Fig. 1. Autoradiograph of rat liver cells incubated with [3H]methapyrine (4.3 μCi; 1 μg/ml) for 24 hr and exposed for 8 weeks. The grains are localized in the cytoplasm of these intact cells. Giemsa, × 1000.

Fig. 2. Autoradiograph of chromosome preparations from rats liver cells treated with [3H]methapyrine and exposed for 8 weeks. Metaphase chromosomes (A: Giemsa, × 1250) and interphase nuclei and surrounding cytoplasm (B: Giemsa, × 1000) do not show any grains over the background.

Fig. 3. Autoradiographs of chromosome preparations from rats liver cells treated with [3H]DMBA (3.7 μCi; 0.025 μg/ml) for 24 hr and exposed for 4 weeks. A number of grains are located on the metaphase chromosomes (A: Giemsa, × 1250). There is a preferential accumulation of grains on the interphase nuclei (B: Giemsa, × 1000). The surrounding cytoplasm also has more grains over the background.
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