Induction of micronuclei and initiation of enzyme-altered foci in the liver of female rats treated with cyproterone acetate, chlormadinone acetate or megestrol acetate

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The synthetic anti-androgen and progestin cyproterone acetate (CPA), recently found to be genotoxic for the liver, and two structurally similar progestins, chlormadinone acetate (CMA) and megestrol acetate (MGA), have been compared for clastogenic and tumor-initiating activities in female rats. In the micronucleus assay, carried out in rats given a single p.o. dose of 100 mg/kg, CPA induced the maximum increase in the frequency of micronucleated hepatocytes (6.6-fold as compared to controls) when treatment was performed 3 days before partial hepatectomy and cell sampling 2 days later. Under the same experimental conditions the clastogenic potencies of CMA and MGA were 69% and 36% of that of CPA respectively. In the liver foci assay, p.o. dosing with 100 mg/kg CPA once a week for 6 successive weeks increased, as compared to controls, a significant increase in the number and area of γ-glutamyltranspeptidase-positive foci. At the same dosage schedule the tumor-initiating activity of CMA and CPA was 10-fold lower than that of CPA. These findings suggest that the 1,2α-methylene group, present in CPA but absent in both CMA and MGA, favours the activation to a reactive species and/or hinders the biotransformation to non-toxic metabolites.

Introduction

Cyproterone acetate (CPA*), a widely used synthetic steroid with both anti-androgenic and progestational activity, was found to increase the incidence of hepatic tumors in rats (1). Subsequent studies suggested that this effect was most likely attributable to tumor-promoting activity (2-4), and this interpretation was favoured by the lack of activity in mutagenicity assays (5). However, in the last few years the following evidence has been given that CPA also has genotoxic activity: it has been shown to form DNA adducts in primary cultures of rat hepatocytes and in the rat liver (6), to elicit DNA repair synthesis in primary hepatocytes from female rats (7), and to induce enzyme-altered foci in the liver of female rats, when administered during the initiation phase (8). More recently CPA has been found to induce DNA repair synthesis in primary cultures of hepatocytes from both male and female human donors (9). To acquire further information about CPA genotoxicity and to compare its effect with that of structurally related progestins we examined in this study the clastogenic and tumor-initiating activities of CPA, chlormadinone acetate (CMA) and megestrol acetate (MGA) in the liver of female rats. Both CMA and MGA differ from CPA for the absence of the 1,2α-methylene group; in addition MGA carries a methyl group at the 6-position where CPA and CMA have a chlorine atom. CMA is a progestogen formerly used with an oestrogen as a 'sequential' oral contraceptive, or as a 'progestogen-only' oral contraceptive. MGA is a progestogen used in the pillative treatment of endometrial and breast cancers.

Materials and methods

Chemicals

CPA, CMA and MGA were purchased from Sigma Chimica (Milan, Italy); N-nitrosodimethylamine (NDMA), and 2-acetylaminofluorene (2-AAF) from Merck (Darmstadt, Germany); 4-acetylaminofluorene from Lancaster Synthesis (MTM Research Chemicals, Eastgate, Morecambe, UK). All other chemicals were of the purest grade available. Animal diets were prepared by Mucedola (Milan, Italy).

Micronucleus assay

The clastogenic activity of the test compounds, as measured by the increase in the frequency of micronucleated hepatocytes, was examined in Sprague-Dawley female albino rats (100-130 g; Harlan-Nossan, Milan, Italy). The experimental design of this assay, based on the model developed by Tates et al. (10-13), is shown in Figure 1. It takes into account both the time dependence of the genotoxic effect of CPA, which in female rats has been found to form the maximum level of liver DNA adducts 3 days after dosing (14), and the opportunity of measuring the clastogenic effect with partial hepatectomy performed before and after the administration of the test compound and with different time intervals between partial hepatectomy and sampling of liver cells.

Rats fasted for 12 h were treated by i.g. gavage with a single dose of 100 mg/kg CPA, CMA or MGA dissolved in 300 μl DMSO. The choice of this dose was based on previous observations about the dose dependence of CPA genotoxic effects (6,8). Negative controls were treated with the same amount of the vehicle. NDMA (10 mg/kg i.p., dissolved in saline), a known hepatocarcinogen, was used as positive control. In addition, taking into account that CPA is a liver mitogen, 4-AAF (500 mg/kg p.o. dissolved in 300 μl DMSO), a strong liver mitogen devoid of genotoxic activity, was used to verify whether the increase in the proportion of dividing cells could produce by itself an increase of micronucleus frequency. Rats treated according to protocol A were given the test compounds 3 days before (two-thirds) partial hepatectomy, and were killed for cell sampling 2 days after partial hepatectomy. Protocol B differed from Protocol A by a 7-day interval between hepatectomy and cell sampling.

Fig. 1. Experimental protocols for the micronucleus assay. T, administration of the test compound; PH, partial (2/3) hepatectomy; S, liver cell sampling.
protocol A only for the 7 day interval between partial hepatectomy and killing. Rats treated according to protocol C were dosed with the test compound 20 h after partial hepatectomy and were killed 3 days later.

The frequencies of micronucleated and binucleated hepatocytes were determined essentially according to Tatematsu et al. (10), using a suspension of liver cells isolated by collagenase perfusion as described by Williams (15). The evidence of a clastogenic effect is evaluated on the basis of micronucleated cells, while an increase, as compared to controls, in the frequency of binucleated hepatocytes indicates that liver cell proliferation induced by partial hepatectomy is reduced due to a toxic effect of the test compound. Data concerning CPA, CMA and MGA were analyzed for the statistical significance of the difference versus corresponding controls by the use of both the Wilcoxon two-sample (two-tailed) test and the Kruskall-Wallis test followed by Dunn’s test for multiple comparisons (16).

Liver foci assay

The tumor-initiating activity of the three progestins was evaluated according to the rat liver assay developed by Tatematsu et al. (17). Fifty-six rats of the same strain, sex and weight of those employed for the micronucleus assay were randomly divided into four groups. CPA, CMA and MGA were dissolved in DMSO immediately before use at a concentration of 20 mg/ml. A dose of 100 mg/kg (0.005 ml/g) was administered by gastric gavage between 9:00 and 10:00 a.m. Rats of groups 2–4 were treated with CPA, CMA and MGA respectively, once a week for 6 successive weeks; rats of group 1 were given the same strain, sex and weight of those employed for the micronucleus assay to the rat liver assay developed by Tatematsu (17). Fifty-six rats of the same strain, sex and weight of those employed for the micronucleus assay were treated with CPA, CMA and MGA.

The results of the liver micronucleus assay carried out in rats given a single oral dose of 100 mg/kg of the three progestins are listed in Table I. In rats treated according to both protocol A and B, CPA induced statistically significant increases in the frequency of micronucleated hepatocytes: 6.6- and 2.6-fold higher than in corresponding controls respectively. In contrast, any evidence of a statistically significant clastogenic response was absent in rats treated according to protocol C, while NDMA, used as positive control, produced under the same experimental conditions the expected increase in the incidence of micronucleated cells.

**Table I. Frequency of micronucleated hepatocytes in rats treated with CPA, CMA or MGA**

<table>
<thead>
<tr>
<th>Treatment conditions</th>
<th>No. of rats</th>
<th>No. of hepatocytes observed</th>
<th>Frequency × 10⁻³ of micronucleated hepatocytes (mean ± SD)</th>
<th>Frequency × 10⁻³ of binucleated hepatocytes (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protocol A</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>4</td>
<td>8695</td>
<td>0.34 ± 0.23</td>
<td>117.7 ± 27.3</td>
</tr>
<tr>
<td>CPA 100 mg/kg p.o.</td>
<td>4</td>
<td></td>
<td>2.24 ± 0.24</td>
<td>208.3 ± 43.2</td>
</tr>
<tr>
<td>CMA 100 mg/kg p.o.</td>
<td>4</td>
<td></td>
<td>1.54 ± 0.27</td>
<td>109.5 ± 21.5</td>
</tr>
<tr>
<td>MCA 100 mg/kg p.o.</td>
<td>4</td>
<td></td>
<td>0.80 ± 0.50</td>
<td>106.0 ± 16.3</td>
</tr>
<tr>
<td>4-AAF 500 mg/kg p.o.</td>
<td>4</td>
<td></td>
<td>0.83 ± 0.43</td>
<td>159.0 ± 23.9</td>
</tr>
<tr>
<td>NDMA 10 mg/kg i.p.</td>
<td>3</td>
<td>5649</td>
<td>13.45 ± 9.77</td>
<td>209.6 ± 72.4</td>
</tr>
<tr>
<td>Protocol B</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>4</td>
<td>8778</td>
<td>0.80 ± 0.23</td>
<td>93.5 ± 14.7</td>
</tr>
<tr>
<td>CPA 100 mg/kg p.o.</td>
<td>4</td>
<td>9472</td>
<td>2.04 ± 1.21</td>
<td>100.5 ± 15.4</td>
</tr>
<tr>
<td>NDMA 10 mg/kg i.p.</td>
<td>4</td>
<td>8229</td>
<td>11.64 ± 3.11</td>
<td>126.6 ± 22.1</td>
</tr>
<tr>
<td>Protocol C</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>4</td>
<td>8316</td>
<td>1.22 ± 0.67</td>
<td>104.0 ± 32.7</td>
</tr>
<tr>
<td>CPA 100 mg/kg p.o.</td>
<td>4</td>
<td>9717</td>
<td>1.33 ± 0.43</td>
<td>141.4 ± 29.8</td>
</tr>
<tr>
<td>NDMA 10 mg/kg i.p.</td>
<td>3</td>
<td>6035</td>
<td>4.31 ± 1.15</td>
<td>67.1 ± 13.2</td>
</tr>
</tbody>
</table>

*Significantly different from control group at P = 0.028 as determined by the Wilcoxon two-sample (two-tailed) test (14).

*Significantly different from control group at P < 0.05 as determined by the Kruskall-Wallis test followed by Dunn’s test for multiple comparisons.

*Excluded from statistical analysis, due to the small sample size.

**Table II. Increase in body weight, liver weight, and GGT-positive foci quantitation in rats initiated with CPA, CMA or MGA (mean ± SD)**

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment conditions</th>
<th>No. of rats</th>
<th>Increase in body wt (g)</th>
<th>Liver wt (% of body wt)</th>
<th>No. of GGT-positive foci (mean ± SD)</th>
<th>Average diameter (μm)</th>
<th>Area (mm²/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>12</td>
<td>151 ± 24</td>
<td>3.40 ± 0.32</td>
<td>0.91 ± 1.83</td>
<td>43 ± 83</td>
<td>141 ± 25</td>
</tr>
<tr>
<td>2</td>
<td>6×100 mg/kg/day CPA</td>
<td>13</td>
<td>168 ± 19</td>
<td>4.31 ± 0.48*</td>
<td>37.37 ± 24.33*</td>
<td>161 ± 1133*</td>
<td>161 ± 57</td>
</tr>
<tr>
<td>3</td>
<td>6×100 mg/kg/day CMA</td>
<td>12</td>
<td>148 ± 14</td>
<td>3.85 ± 0.34*</td>
<td>40.50 ± 4.17*</td>
<td>223 ± 226</td>
<td>138 ± 55</td>
</tr>
<tr>
<td>4</td>
<td>6×100 mg/kg/day MGA</td>
<td>12</td>
<td>157 ± 30</td>
<td>3.49 ± 0.17</td>
<td>5.08 ± 5.38</td>
<td>240 ± 255</td>
<td>144 ± 34</td>
</tr>
</tbody>
</table>

*Statistical analysis was performed by use of ANOVA followed by the Student–Newman–Keuls test for multiple comparisons (a) and the Kruskall-Wallis test followed by Dunn’s test for multiple comparisons (b). *Significantly different from group 1 at P < 0.05.
On the basis of these findings the clastogenic effect of CMA and MGA was examined only according to protocol A (Table I). With a single oral dose of 100 mg/kg CMA, the average frequency of micronucleated hepatocytes was 4.5-fold higher than in corresponding controls. This response, which should be considered biologically relevant, was statistically significant according to the Wilcoxon two-sample (two-tailed) test, but not according to the Kruskall–Wallis test followed by Dunn’s test for multiple comparisons, which is generally considered a more appropriate approach for the analysis of this type of data (16). At the same dosage schedule, MGA induced a 2.3-fold increase in the frequency of micronuclei, but this response is of doubtful biological relevance as it was not statistically significant according to both the methods reported above. In rats treated according to the same protocol A, a non-significant increase of micronucleated hepatocytes was observed with 4-AAF (500 mg/kg p.o.), the strongest non-carcinogenic liver mitogen yet recorded (21), and there was the expected strong clastogenic response with NDMA (10 mg/kg i.p.) used as positive genotoxic control.

The frequency of binucleated hepatocytes, which should tend to disappear after partial hepatectomy, was increased in rats treated with CPA and NDMA according to protocol A; these findings suggest a toxicity-induced delay in the progress of liver regeneration.

Liver foci assay
The results obtained are listed in Table II. During the experimental period a few rats died in all the four groups (two in group 1, one in group 2, two in group 3 and two in group 4); since death occurred within a few days after partial hepatectomy, it should be considered as due to postoperative complications rather than to toxicity. None of the three progestins induced a statistically significant change of body weight increase. Liver weight of rats treated with CPA was significantly increased as compared to both controls and rats treated with CMA or MGA.

The quantitative analysis of GGT-positive foci in comparison with the control group revealed that their average number and area were markedly increased by CPA administered p.o. at the dose of 100 mg/week for 6 successive weeks. In contrast, only a modest increase of the same parameters, which did not reach the level of statistical significance because of the large inter-animal variability, was observed in rats treated with the same dose of CMA and MGA. In this respect it is worth noting that a positive response, i.e. the presence of GGT-positive foci, was observed in 12/13 rats given CPA, 8/12 rats given CMA and MGA, and only in 3/12 control rats. Basophilic foci were absent in rats of all the four groups.

Discussion
CPA, previously found to behave as a sex steroid genotoxic for the liver (6–9), has been compared with two structurally similar progestins, CMA and MGA, for the capacity to induce a clastogenic effect, as measured by an increase in the frequency of micronucleated hepatocytes, and to initiate tumor growth, as evaluated by the induction of enzyme-altered preneoplastic foci in the liver. The study was carried out in female rats, since the genotoxic activity of CPA has been shown to be markedly higher in female than in male rats.

In the micronucleus test, CPA produced a positive response when administer 3 days before partial hepatectomy, consistently with the occurrence, 3 days after dosing, of the maximum DNA adduct level (14). The negative response observed with 4-AAF, a very potent liver mitogen devoid of hepatocarcinogenic activity, suggests that the clastogenic effect of CPA is independent of its mitogenic activity. On the other hand, the increased frequency of binucleated cells indicates that in this case CPA did not stimulate hepatocyte proliferation. Under the same experimental conditions the clastogenic potency of CMA was lower (69%) than that of CPA but still biologically significant, whereas the response observed with MGA should be considered negative or at least equivocal.

In the liver foci assay, CPA displayed, as judged by the induction of GGT-positive foci, a clear initiating activity, in good agreement with the results previously obtained using a different experimental model by Deml et al. (8); in contrast, both CMA and MGA produced only a very modest and not statistically significant increase of GGT-positive foci, which, depending on the parameter considered, was 7- to 10-fold lower than that produced by CPA. It is worth noting the absence, for all the three progestins, of the hyperplastic basophilic foci that Tatematsu et al. (17), using the same experimental model, observed in the liver of rats exposed to several known carcinogens. Moreover, the tumor-initiating activity of CPA was weaker than that observed by Tatematsu et al. (17) in rats initiated with N-nitrosodiethylamine (10 mg/kg weekly for 7 successive weeks) and then placed for 2 weeks on a diet containing 2-AAF.

The results of this study and previous findings provide evidence that CPA, CMA and MGA are characterized by different profiles of genotoxic activity. CPA has been found to behave as a genotoxic steroid for three of the endpoints usually examined: DNA damage, chromosome alterations and tumor-initiating activity. In contrast, less uniform responses have been observed with CMA and MGA. Both have been shown to be very weakly positive, as compared with CPA, in terms of liver DNA adducts and negative in terms of DNA repair measured by the BrdUrd density-shift method (22), whereas experiments still in progress (A.Martelli et al., unpublished data) indicate that CMA and MGA elicit autoradiographic DNA repair synthesis with a potency very similar to that of CPA in primary cultures of hepatocytes from human donors of both genders and from female but not from male rats; at present the meaning of this puzzling discrepancy cannot be explained. In this study CMA has been found to induce chromosome alteration but to a lower extent than CPA, and MGA was in this respect inactive or only marginally active (Table I); moreover, both CMA and MGA displayed, in comparison with CPA, a very weak tumor-initiating activity (Table II). Unfortunately the epidemiological data on the incidence of liver tumors in women who had taken drugs that contain these three progestins are so far very limited. As a consequence it is difficult to establish to what extent genotoxicity data can be considered indicative of a different role played by CPA, CMA and MGA in human liver carcinogenesis. However, it is worth noting that four cases of hepatocellular carcinoma among CPA users (23,24) and one case among CMA users (25) have been already reported.

Schwarz et al. (26) formulated the hypothesis that CPA is reduced to the 3-OH derivative and then sulfated at this position by hydroxysteroid-sulfotransferase, forming a labile sulfate ester; the subsequent spontaneous elimination of the sulfate group gives rise to a reactive carbonium ion. This hypothesis is consistent with the recent identification of 3α-hydroxy-CPA as a metabolite of CPA in the bile of female
rats and with the potential of this metabolite to form DNA adducts in vitro (27). CMA and MGA differ from CPA for the absence of the 1,2 α-methylene group; in addition MGA carries a methyl group at the 6-position, whereas CPA and CMA have a chlorine atom. To explain the comprehensive lower genotoxic activity of CMA and MGA it may be tentatively hypothesized that the 1,2 α-methylene group present in the CPA molecule favours its reduction to the 3-OH derivative and/or the formation of the labile sulfate ester. Alternatively, the 1,2α-methylene group might hinder the biotransformation of CPA to non-toxic metabolites; this hypothesis is consistent with the observation that CMA is metabolized faster than CPA in primary cultures of hepatocytes from female rats (Schering AG, unpublished results).

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References

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