Tumor-promoting Activity of 2,3-Dihydrophorbol Myristate Acetate and Phorbol Myristate Acetate in Mouse Skin¹

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ABSTRACT

Phorbol myristate acetate (PHMA) had been previously prepared from the potent mouse skin tumor promoter phorbol myristate acetate (PMA) by sodium borohydride reduction of the C-5 carbonyl group in PMA to a secondary alcohol. PHMA was shown to have an inflammatory effect in mouse skin equal to that of PMA. 2,3-Dihydrophorbol myristate acetate (DPMA), a new compound, was prepared from the 3-aldheyde of PMA by catalytic hydrogenation. DPMA exhibited no detectable inflammatory effect in mouse skin. Both DPMA and PHMA were tested on the dorsal skins of female ICR/Ha Swiss mice (30/group) for 433 and 380 days, respectively, in separate experiments. The tumor-promoting activity of both compounds was reduced significantly, compared with that of equimolar doses of PMA. For each treatment the number of mice with tumors per total number of tumors was: DPMA, 9/17; PMA, 29/553 at 10 μg/mouse; PHMA, 30/317; PMA, 24/69 at 2.5 μg/mouse. The results suggest that specific binding requirements influence the tumor-promoting and hyperplastic activity of PMA and its closely related derivatives in mouse skin.

INTRODUCTION

Of the various esters of the diterpene alcohol phorbol, which have been isolated from croton oil, the diester PMA²,³ (Chart 1) is the most potent promoter of 2-stage carcinogenesis in mouse skin. The isolation and structural characterization of PMA as well as its biochemical and morphological effects on mouse skin have been the subject of several recent reviews (2, 7, 14). Also, it was recently reported that PMA is metabolized to PHMA (Chart 1) in mouse skin (10). Applied to mouse skin, PHMA was shown to produce as much inflammation as did an equimolar dose of PMA, 28 hr after application (10). In the present work PHMA showed a decrease in tumor-promoting activity in the total number of tumors compared to that with PMA, and these results stimulated further work on the effects of structural changes in PMA on tumor-promoting and inflammatory activity in mouse skin. In the present report the synthesis of DPMA is described, and the comparative tumor-promoting activities of DPMA, PHMA, and PMA in mouse skin are given. A preliminary report concerning this work has appeared in the literature (11).

MATERIALS AND METHODS

Bioassay. Female ICR/Ha Swiss mice (A. R. Schmidt, Madison, Wis.) were used for the experiments. Two different shipments provided mice for the experiments comparing the tumor-promoting activity in mouse skin of DPMA with PMA and of PHMA with PMA. The animals, 8 weeks old at the beginning of the experiments, were housed 6/cage on sterile wood chips (Iso-Dri; Fisher and Son, Bound Brook, N. J.), fed Purina laboratory chow and water ad libitum, and weighed regularly. The animal rooms were maintained at 22-24°.

The dorsal skins of mice were clipped free from the hair the day before the initial treatment and then as needed for the duration of the experiment. The solutions were all applied by micropipet to the clipped areas (~1 sq cm): a single treatment with 20 μg 7,12-dimethylenz(a)anthracene in 0.1 ml acetone was followed 2 weeks later by applications 3 times/week of the test compounds in 0.1 ml acetone. The dosages and the duration of the experiments are given in Tables 1 and 2. Equivalent doses of DPMA, PHMA, and PMA are approximately equimolar. The scoring and histopathology were carried out as described in our earlier report (17).

Inflammatory Effects of DPMA in Mouse Skin. Five mice/group were used. DPMA (10 μg/0.1 ml acetone) was applied to the dorsal skin once. Mice were killed 28 hr after the application of DPMA for reasons explained in our previous report (10), and skin specimens were fixed in 4% formalin, blocked in paraffin, sectioned at 4 μm, and stained with hematoxylin and eosin. Inflammatory effects were based on the criteria used in a previous work (17) in which dermal cellular infiltration and interfollicular epidermal hyperplasia were evaluated.

Chemicals. Dihydrogen hexachloroplatinate(IV) was purchased from Alfa Division, Ventrón Corp., Danvers, Mass. Sodium nitrite was obtained from Matheson, Coleman & Bell, East Rutherford, N. J. Sodium nitrate was obtained from Fisher Scientific Co., Springfield, N. J. Platinum oxide was prepared as described before (4). PAMA, PHMA, and PMA were prepared as described in previous reports from this laboratory (10, 17). The preparation of DPMA and its derivative are described separately below.

Column Chromatography and TLC. Column chromatography was carried out on columns (1.5 x 24 cm) of Silica Gel 60 (70 to 230 mesh) obtained from Brinkmann Instruments, Inc., Westbury, N. Y. Analytical TLC was performed...
Table 1

<table>
<thead>
<tr>
<th>Primary treatment</th>
<th>Secondary treatment</th>
<th>Dose (μg)</th>
<th>Time to first papilloma (days)</th>
<th>Mice with papillomas/total papillomas</th>
</tr>
</thead>
<tbody>
<tr>
<td>7,12-Dimethylbenz(a)anthracene</td>
<td>PMA</td>
<td>2.5</td>
<td>51</td>
<td>30/317(^c) (10)(^d)</td>
</tr>
<tr>
<td>7,12-Dimethylbenz(a)anthracene</td>
<td>PHMA</td>
<td>2.5</td>
<td>55</td>
<td>24/69 (0)</td>
</tr>
<tr>
<td>7,12-Dimethylbenz(a)anthracene</td>
<td>PHMA</td>
<td>10</td>
<td>54</td>
<td>29/181 (10)</td>
</tr>
<tr>
<td>None</td>
<td>PMA</td>
<td>2.5</td>
<td>169</td>
<td>2/2 (0)</td>
</tr>
<tr>
<td>None</td>
<td>PHMA</td>
<td>2.5</td>
<td>380</td>
<td>2/2 (1)</td>
</tr>
<tr>
<td>None</td>
<td>PHMA</td>
<td>10</td>
<td>380</td>
<td>2/2 (0)</td>
</tr>
<tr>
<td>7,12-Dimethylbenz(a)anthracene</td>
<td>Acetone</td>
<td></td>
<td></td>
<td>0</td>
</tr>
</tbody>
</table>

\(^a\) One application only of 20 μg/0.1 ml acetone to dorsal skin.

\(^b\) Applications 3 times/week, beginning 14 days after primary treatment for the duration of the test in 0.1 ml acetone/application.

\(^c\) Median survival time for this group was 350 days; 1 additional animal bore a fibrosarcoma.

\(^d\) Numbers in parentheses, mice with squamous carcinoma.

Instrumentation. UV spectra were recorded in 95% ethanol in 1-cm cells in a Beckman Model 25 spectrophotometer. IR spectra were recorded in KBr pellets in a Perkin-Elmer 137 spectrophotometer. NMR spectra were recorded in a Perkin-Elmer R32 90-MHz spectrometer (Baron Consulting Co., Orange, Conn). EIMS were recorded in a Hitachi Perkin-Elmer RMU-6-D mass spectrometer at 70 eV and at a source temperature of 150° (Morgan-Schaefer Corp., Montreal, Quebec, Canada). CIMS were obtained in this laboratory with a Dupont 21-492 high-resolution mass spectrometer at 70 eV and at a source temperature of 275°. CIMS were obtained with Matheson instrument-grade (99.5%) isobutane maintained at ~0.5 torr. Samples for CIMS were admitted to the ionization chamber by direct
exhibited a small molecular ion peak at m/e 618, which is absent in the NMR spectrum of DPMA. The EIMS of DPMA acid), and the base peak was observed at m/e 330 (loss of 540 (loss of acetic acid + H2O), and 390 (loss of myristic acid). The molecular weight of DPMA (C37H60O4). Major peaks were 619 (M + H)⁺, but it did give addition ions at m/e 659 [618 + 41⁺ (C7H15O)⁺] and 661 [618 + 43⁺ (C9H17O)⁺] (8, 12). Additional peaks consistent with the fragmentation of a protonated molecular ion at m/e 619 were at 601 (loss of 1 H2O), 583 (loss of 2 H2O). 559 (small peak; loss of acetic acid), 541 (loss of acetic acid + 1 H2O), 523 (loss of acetic acid + 2 H2O), 391 (base peak; loss of myristic acid), 373 (loss of myristic acid + 2 H2O), 331 (loss of acetic and myristic acids), 313 (loss of acetic and myristic acids + 1 H2O), 295 (loss of acetic and myristic acids + 2 H2O), and 229 [protonated myristic acid (C15H29O2)⁺]. The mass spectra of a relevant series of other phorbol derivatives obtained in this laboratory are described elsewhere (12).

Preparation of the 4-Carboxy-4'-nitroazobenzene Derivative of DPMA. The preparation of the 4-carboxy-4'-nitrophenoxyazobenzene derivative of DPMA was carried out as described previously (6, 16). The product was chromatographed on a column with Solvent System E; the desired derivative was eluted from the column in the first 75 ml of eluate and was crystallized from methanol [m.p. 119–121°C, Rf (Solvent System F), 0.48].

C15H29O4N2
Calculated: N 4.8
Found: N 4.5

RESULTS

Chemistry. The structure of DPMA (Chart 1), the product obtained in 50% yield from the catalytic hydrogenations of PMA (Chart 1), was deduced from IR, UV, NMR, CIMS, and EIMS as well as the preparation of a crystalline 4-carboxy-4'-nitrophenoxyazobenzene derivative. The IR spectrum of DPMA was similar to that of PMA (Chart 1); the UV absorption at 241 nm and the NMR signal at 7.60 ppm (C-2 olefinic proton) also supported the presence of an α-β-unsaturated carbonyl chromophore in the 5-membered ring of DPMA. The reduction of the 2,3-double bond in DPMA was supported by the absence of a C-2 olefinic proton signal present at 5.56 ppm in the NMR spectrum of PMA and PHMA (Chart 1) and at 5.74 ppm in phorbol triacetate (3). The CIMS and EIMS confirmed the assigned structure. The ability of DPMA to form a 4-carboxy-4'-nitrophenoxyazobenzene derivative confirms the presence of the primary 3-hydroxymethyl group.

Tumor-promoting Activity. PHMA was tested at 2.5 and 10 μg in mouse skin (Table 1 and Chart 2), and PMA was tested at 2.5 μg. The times in days to first papilloma of PHMA at the 2 doses and of PMA were the same, 51 to 54 days, whereas the number of mice with papillomas was 29 of 30 for PHMA at 10 μg, 24 of 30 for PHMA at 2.5 μg, and 30 of 30 for PMA at 2.5 μg. The total number of papillomas was reduced from 317 for PMA at 2.5 μg to 69 for PHMA at 2.5 μg, a decrease of 78%. The total number of papillomas was 181 for PHMA at 10 μg. The control groups showed the expected lower tumor incidences as shown in Table 1. No skin tumors were observed in control groups that received...
with 10 ng PMA (O), 2.5 Mg PMA (•), 10 Mg PHMA (D), 2.5 Mg PHMA (A), 10 Mg DPMA (A).

acetone only or no treatment. These are not shown in Table 1.

When DPMA was applied to mouse skin once only at a dose of 10 μg/mouse it produced no observable inflammatory effects after 28 hr (10) as determined by the same criteria used in previous studies (17). DPMA was tested for tumor-promoting activity in mouse skin at 10 μg/dose 3 times/week and compared with PMA at the same dose (Table 2). The time in days to first papilloma was 220 for DPMA compared with 49 days for PMA, whereas the number of mice with papillomas was 9 of 30 for DPMA and 29 of 30 for PMA. The total number of tumors was 17 for DPMA and 590 for PMA. No skin tumors were observed in control groups that received acetone only or no treatment. These are not shown in Table 2. Chart 2 illustrates the rate of appearance of tumors for DPMA, PHMA, and PMA.

**DISCUSSION**

Chemistry. DPMA was prepared in 50% yield from PMA by a catalytic hydrogenation procedure with the addition of sodium nitrite (4). Previous attempts at catalytic hydrogenation of phorbol triacetate (ester groups at C-9, 9a, and 3-hydroxymethyl) with palladium on charcoal or phorbol with platinum as catalyst resulted in hydrogenation of Δ^{2,3} and Δ^{6,7} as well as hydrogenolysis of the allylic acetoxy (3-acetoxyethyl) or allylic hydroxy (3-hydroxymethyl) to yield the 3-methyl group (7). The structure of DPMA was assigned on the basis of IR, UV, NMR, CIIMS, EIIMS, and the preparation of a crystalline 4-carboxy-4'-nitrophenylazobenzene derivative. The configuration (α and β) of the 3-hydroxymethyl groups and of its axial or equatorial position was not established. The UV of DPMA gave an absorption maximum at 241 nm. UV spectra of PMA, phorbol, and many of its derivatives taken in various solvents have given absorption maxima between 230 and 235 nm (3, 5, 13).

The mechanism for the reduction in mouse skin of the C-5 carbonyl group in PMA to a secondary alcohol (10) may be ascribed to a class of enzymes known as cytoplasmic aldoketoreductase, which are widely distributed in tissues; they are known to metabolize aldehyde and ketone groups to alcohols in various types of molecules, some of which are comparable to PMA in complexity of structure (1).

Structure Activity Relationships. Reduction of the carbonyl group in the C-5 of PMA to a secondary hydroxyl group causes both a functional change in groups on the phorbol ring and a small stereochemical change (a decrease in planarity of the 5-membered ring) as shown by Dreiding models. This results in decreased tumor-promoting activity of PHMA compared to PMA at equimolar doses in the total number of mice with papillomas and carcinomas and tumor multiplicity (Table 1 and Chart 2), but it is noteworthy that the times in days to first tumors were very similar for the 3 groups concerned. Also, PHMA, although it is less potent than PMA as a promoting agent, retains its irritant activity. The relationship between irritancy and tumor promotion remains unclear in spite of numerous studies and reviews (2, 13, 14), and there is in fact some evidence to suggest that hyperplasia and skin damage is not related at all to promoting activity (9, 14). Saturation of the 2,3-double bond PMA gave DPMA (Chart 1). Structurally, DPMA differs both functionally and stereochemically from PMA. The functional differences are important in that in PMA the 3-hydroxymethyl group is allylic and thus differs in reactivity from the 3-hydroxymethyl group attached to a saturated C-3 carbon atom in DPMA. As shown by Dreiding models, the saturated 7-membered ring in DPMA exhibits much greater conformational flexibility than does the corresponding 7-membered ring in PMA, which contains a Δ^{2,3} double bond. DPMA shows weak tumor-promoting activity compared to that of PMA and PHMA and shows no observable inflammatory effects by light microscopy (Table 2 and Chart 2) at equimolar doses. Significantly, the time in days to first tumor with this compound is markedly late, compared to the other 2 compounds. (Parenthetically, the time in days to first papilloma is significant in that many assays for tumor-promoting activity of chemical compounds have been conducted for 26 weeks or less (2, 7) and negative results from some of these experiments are undoubtedly invalid.)

The decrease in tumor-promoting activity in PHMA and DPMA compared with PMA suggests a specific binding of promoter to receptor(s) in initiated cells. Recently, evidence was presented suggesting that receptors for PMA exist in the plasma membranes of cells (15). Exposure of membranes to PMA caused up to a 21% decrease of the native membrane emission, whereas treatment of the membranes with PHMA resulted in a 5 to 10% decrease. It is not known at the present time whether receptor sites for PMA, which are crucial to the process of tumor promotion in mouse skin, are present in both normal and initiated skin or whether these receptors are unique to the initiated cell.

Studies are currently underway on the tumor-promoting activity of PAMA and several 4α-stereoisomers of PMA (B. L. Van Duuren, unpublished data). The 4α-stereoisomers were recently synthesized in this laboratory (12). A knowledge of structure-activity relationships of the compounds discussed in this report and of related compounds will be valuable in future biochemical studies on the mode of action of PMA and related compounds.
REFERENCES

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