Characterization of the antitumor-promoting activity of camptothecin in SENCAR mouse skin

Xiao Mei Gao, Elisabeth M. Perchellet, Amy W. Davis, Steven W. Newell, Duy H. Hua and Jean-Pierre Perchellet

Abstract

(+)-Camptothecin (CPT), a topoisomerase I inhibitor specifically toxic toward S phase cells, was tested topically for its ability to inhibit skin tumor initiation by 7,12-dimethylbenz[a]anthracene (DMBA) and complete tumor promotion by 12-O-tetradecanoylphorbol-13-acetate (TPA) in SENCAR mice. Even though CPT does not prevent the covalent binding of a subcarcinogenic dose of DMBA to DNA, it enhances early inhibition of DNA synthesis caused by this initiator and may decrease the essential role of DNA replication in tumor initiation. Indeed, CPT (400 nmol) applied 4 h before or 1 h after TPA inhibits the yield, but not the incidence, of skin tumors initiated by this compound. Moreover, because it inhibits TPA-stimulated DNA synthesis at 16 h when applied 12 h after the tumor promoter, CPT partially decreases tumor initiation when DMBA is applied 16 h after a TPA pre-treatment. CPT (400 nmol) applied 1 h before or 4, 12, 24 or 48 h after each promotion treatment with TPA remarkably inhibits the incidence and yield of skin tumors promoted by this agent. CPT delays and inhibits promotion of skin tumors the most when applied 12–24 h after each TPA treatment, at times when it can block the stimulation of DNA synthesis that follows the period of early inhibition caused by TPA. The ability of post-treatments with 25, 100 and 400 nmol CPT to inhibit skin tumor promotion is dose dependent. In the TPA (stage 1)–mezerein (stage 2) protocol CPT (400 nmol) post-treatment inhibits both the first and second stages of tumor promotion, related to its ability to decrease the DNA and ornithine decarboxylase responses required for stages 1 and 2 respectively. The classic model of multistage skin carcinogenesis, therefore, may be valuable to determine if novel CPT analogs are more effective than their parent compound at inhibiting tumor initiation, promotion and progression.

Introduction

The sequential steps of skin carcinogenesis include tumor initiation, stage 1 (conversion) and stage 2 (propagation) promotion and progression (1–3). Because the mutagenic events of tumor initiation are irreversible, it is fundamental to the prevention of neoplasia to identify agents that are effective against the reversible propagation phase of tumorigenesis. 20(S)-Camptothecin (CPT*) is a topoisomerase I (Topo I) inhibitor that exhibits a broad spectrum of antitumor activity (4,5). Since Topo I inhibitors may be mutagenic in vitro (6), it is critical to elucidate their mechanism of anticarcinogenesis and to characterize their desired and undesired effects during the multistage process of tumorigenesis.

Recently we found that CPT fails to alter the covalent binding of an initiating dose of 7,12-dimethylbenz[a]anthracene (DMBA) to epidermal DNA in vivo (7). However, CPT significantly inhibits stimulation of DNA synthesis by the tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) and a carcinogenic dose of DMBA in mouse epidermis in vivo, even when applied several days after these agents (7,8). CPT may indirectly decrease the ornithine decarboxylase (ODC)-inducing activity of multiple TPA treatments, because it can inhibit stimulation of RNA synthesis by this compound (7,8). In contrast, CPT fails to prevent TPA-stimulated hydroperoxide production, due to its inability to inhibit TPA-stimulated protein synthesis (7,8).

The two-step model of carcinogenesis in mouse skin provides a means to distinguish between the anti-initiating and the antitumor-promoting effects of the anticancer drugs tested, thereby providing valuable clues about their mechanisms of action (1–3). Since CPT inhibits several biochemical markers of tumor promotion (7,8) and may decrease the role of DNA replication during tumor initiation, the present study was undertaken to determine if this Topo I inhibitor specifically alters tumor initiation and complete or two-stage tumor promotion.

Materials and methods

TREATMENT OF MICE

Female SENCAR mice, 5 weeks old, from Harlan Sprague Dawley Inc. (Indianapolis, IN), were housed and maintained and their dorsal skin shaved before experimentation (9). Solutions of DMBA (Eastman Kodak Co., Rochester, NY), mezerein (MEZ) and TPA (both from LC Laboratories, Woburn, MA) were delivered to the shaved backs of mice in 0.2 ml acetone. The dose of 400 nmol CPT (Sigma Chemical Co., St Louis, MO) was applied topically in 0.4 ml dimethyl sulfoxide (DMSO):acetone (1:9) at the appropriate times before or after each application of tumor initiator or promoter and to the same area of skin. This solution was further diluted with acetone to administer the lower doses tested. Controls were similarly treated with acetone and vehicle only and, in every experiment, all mice received the same volume of solvent.

DETERMINATION OF DNA SYNTHESIS

The rate of incorporation of [methyl-3H]thymidine (51 Ci/mmol; Amersham Corp., Arlington Heights, IL) into epidermal DNA was determined in groups of three mice sacrificed 6 or 16 h after treatment with TPA or an initiating dose of DMBA (10–12). The mice received an i.p. injection of 30 μCi [3H]thymidine 40 min before the indicated times of sacrifice. Control mice treated only with acetone and vehicle were sacrificed after the same 40 min period of pulse labeling. Epidermal homogenates were prepared from each mouse, the macromolecules were precipitated by acidification with HClO4 and the acid-insoluble pellets were washed essentially as described previously (12). DNA was hydrolyzed from the precipitate with 3 ml 0.5 N HClO4 for 15 min at 90°C. The radioactivity incorporated in each sample was estimated

ABBREVIATIONS: CPT, camptothecin; Topo I, topoisomerase I; DMBA, dimethylbenz[a]anthracene; TPA, 12-O-tetradecanoylphorbol-13-acetate; ODC, ornithine decarboxylase; MEZ, mezerein; DMSO, dimethyl sulfoxide; PAs, papillomas; SSBs, single-strand breaks; DSBs, double-strand breaks; PKC, protein kinase C.
Table I. Ability of CPT to inhibit DNA synthesis in the presence and absence of a tumor initiator or promoter in mouse epidermis in vivo

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>Epidermal DNA synthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>at +6 h</td>
</tr>
<tr>
<td></td>
<td>c.p.m./μg DNA ± SD (n = 3)</td>
</tr>
<tr>
<td>Control (0 h)</td>
<td>35.9±4.3</td>
</tr>
<tr>
<td>+ CPT (-1 h)</td>
<td>24.9±2.5b</td>
</tr>
<tr>
<td>+ CPT (+1 h)</td>
<td>25.4±2.2b</td>
</tr>
<tr>
<td>+ CPT (+12 h)</td>
<td>18.8±1.9</td>
</tr>
<tr>
<td>DMBA (0 h)</td>
<td>10.8±0.9f</td>
</tr>
<tr>
<td>+ CPT (+1 h)</td>
<td>20.2±2.4</td>
</tr>
<tr>
<td>TPA (0 h)</td>
<td>9.9±0.8d</td>
</tr>
<tr>
<td>+ CPT (+1 h)</td>
<td>260.5±30.7e</td>
</tr>
</tbody>
</table>

*Doses applied: CPT, 400 nmol; DMBA, 20 nmol; TPA, 5 nmol. The time of application in relation to that of DMBA or TPA treatment is given in parentheses.

**P < 0.025, significantly smaller than control.

***P < 0.005, significantly smaller than DMBA.

†P < 0.005, significantly smaller than TPA.

Not significantly different from control.

<table>
<thead>
<tr>
<th>Group</th>
<th>Tumor initiation by DMBA (1 × at time 0)</th>
<th>Complete tumor promotion by TPA (2×/week at time 0)</th>
<th>Week of 1st PA</th>
<th>Observations at week 20</th>
<th>Mouse wt (g)</th>
<th>Percent survival</th>
<th>Tumor wt/mouse mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DMBA</td>
<td>TPA</td>
<td>6</td>
<td>39.4</td>
<td>97</td>
<td>639</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>+ TPA (-16 h)</td>
<td>TPA</td>
<td>4</td>
<td>39.5</td>
<td>94</td>
<td>549</td>
<td>86</td>
</tr>
<tr>
<td>3</td>
<td>+ TPA (-16 h) + CPT (400 nmol, -4 h)</td>
<td>TPA</td>
<td>4</td>
<td>40.0</td>
<td>97</td>
<td>341</td>
<td>53</td>
</tr>
<tr>
<td>4</td>
<td>+ CPT (400 nmol, -4 h)</td>
<td>TPA</td>
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<td>40.1</td>
<td>100</td>
<td>425</td>
<td>67</td>
</tr>
<tr>
<td>5</td>
<td>+ CPT (400 nmol, +1 h)</td>
<td>TPA</td>
<td>6</td>
<td>39.1</td>
<td>97</td>
<td>406</td>
<td>64</td>
</tr>
<tr>
<td>6</td>
<td>DMBA</td>
<td>+ CPT (400 nmol, -1 h)</td>
<td>7</td>
<td>36.9</td>
<td>90</td>
<td>32</td>
<td>5</td>
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<tr>
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<td>DMBA</td>
<td>+ CPT (400 nmol, +4 h)</td>
<td>7</td>
<td>37.8</td>
<td>84</td>
<td>25</td>
<td>4</td>
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<tr>
<td>8</td>
<td>DMBA</td>
<td>+ CPT (25 nmol, +12 h)</td>
<td>5</td>
<td>37.5</td>
<td>100</td>
<td>461</td>
<td>72</td>
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<tr>
<td>9</td>
<td>DMBA</td>
<td>+ CPT (100 nmol, +12 h)</td>
<td>7</td>
<td>35.7</td>
<td>100</td>
<td>68</td>
<td>11</td>
</tr>
<tr>
<td>10</td>
<td>DMBA</td>
<td>+ CPT (400 nmol, -4 h)</td>
<td>11</td>
<td>38.4</td>
<td>87</td>
<td>12</td>
<td>2</td>
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<tr>
<td>11</td>
<td>DMBA</td>
<td>+ CPT (400 nmol, +12 h)</td>
<td>13</td>
<td>37.6</td>
<td>94</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>12</td>
<td>DMBA</td>
<td>+ CPT (400 nmol, +48 h)</td>
<td>9</td>
<td>38.9</td>
<td>94</td>
<td>45</td>
<td>7</td>
</tr>
</tbody>
</table>

*The conditions of the tumor experiments are detailed in Figures 1–4.

†Expressed as a percentage of group 1.

The results were expressed as the percentage of survivors bearing papillomas (PAs) and the average numbers of PAs/survivor. Statistics for the differences between the means of PAs/mouse were performed using the normal approximation of the Mann–Whitney U test, whereas differences between PA incidences were compared using the χ² statistic (13). The level of significance in both cases was set at P < 0.05.

Results
In contrast to a carcinogenic dose, an initiating dose of DMBA causes a transient inhibition of DNA synthesis that is not followed by a prolonged period of stimulation (10,11). Indeed, epidermal DNA synthesis is inhibited by 48% at +6 h after DMBA initiation at time 0 and returns to the control level at +16 h (Table I). When applied at +1 h a post-treatment with 400 nmol CPT decreases basal DNA synthesis and reduces even further the DMBA-inhibited level of DNA synthesis at +6 h, but does alter DNA synthesis at +16 h (Table I).

A single dose of 400 nmol CPT applied 4 h before or 1 h after the time of tumor initiation with 10 nmol DMBA fails...
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Fig. 1. Effects of CPT pre- and post-treatments on skin tumor initiation. Skin tumors were initiated in all mice by a single application of 10 nmol DMBA. One week later all mice were promoted twice a week with 3.24 nmol TPA for the rest of the experiment. The dose of 400 nmol CPT was applied topically 4 h before (D) or 1 h after (•) the single initiation treatment with DMBA (A) at time 0. (A) Average number of PAs/survivor. (B) Percentage of survivors with PAs.

to delay or inhibit the incidence of skin tumors subsequently promoted by TPA (Figure 1B). However, CPT treatment, especially after DMBA initiation, slightly reduces the average number (Figure 1A) and weight (Table II, groups 4 and 5) of skin tumors/mouse, perhaps because CPT decreases critical DNA replication occurring after the interaction of DMBA with DNA but before the initial DNA damage is repaired (14-16).

The DNA response to TPA is characterized by an 8 h period of early inhibition preceding a prolonged period of stimulation that first peaks at 16 h (10,12). When applied at -1 h a pre-treatment with 400 nmol CPT decreases basal DNA synthesis and reduces even further the level of DNA synthesis already inhibited by 44% at 6 h after TPA treatment at time 0 (Table I). Such CPT pre-treatment, however, cannot prevent the 9-fold elevation in DNA synthesis caused by TPA at +16 h. In contrast, a CPT post-treatment at +12 h decreases basal DNA synthesis at +16 h and inhibits by 83% the peak stimulation of DNA synthesis occurring +16 h after TPA treatment (Table II). As compared with controls, no differences were noted in the cellular morphology of the epidermis treated with CPT in the absence and presence of DMBA or TPA treatments, thereby ruling out any local toxicity caused by the drug alone or in combination with the initiator or promoter (data not shown). Since TPA-stimulated DNA synthesis normally occurs after CPT pre-treatment, the early depression of basal and TPA- or DMBA-inhibited DNA synthesis by CPT is unlikely to cause toxic effects limiting subsequent epidermal responses (Table I).

Because it blocks the increase in epidermal DNA synthesis
To the best of our knowledge, no antitumor promoter has been discovered that can inhibit complete tumor promotion when applied at times when most of the biochemical markers of tumor promotion have already been maximally stimulated. Thus an exciting facet of this work was to determine if the CPT post-treatments that blocked the DNA response to TPA in our preliminary studies (7,8) were capable of inhibiting tumor promotion even when applied 12–48 h after each TPA or MEZ treatment. As shown in Figure 3 and Table II (groups 6 and 7 and 10–12), 400 nmol CPT applied at five different times in relation to each TPA treatment dramatically inhibit complete tumor promotion and reduce the average tumor weight/mouse by 93% or more. However, CPT is clearly a more effective antitumor promoter when it is applied 12 or 24 h after rather than 1 h before or 4 or 48 h after each promotion treatment (Figure 3). In the control mice promoted only with TPA the first PAs appear at 6 weeks and start to plateau at 10 weeks, with ~93% of the mice bearing PAs and an average of more than 10 PAs/mouse (Figure 3). At this stage the mice protected by CPT 12 or 24 h after each promotion treatment with TPA had not developed a single tumor. In those CPT-protected groups the first PAs appeared after 11 and 13 weeks and their final tumor incidence, yield and weight/mouse at 20 weeks were inhibited by at least 71, 96 and 98% respectively (Figure 3 and Table II, groups 10 and 11). At 400 nmol, therefore, CPT post-treatments remarkably delay and inhibit the complete process of skin tumor promotion by TPA. CPT inhibits tumor promotion in a dose-dependent manner when applied 12 h after each TPA treatment (Figure 4). Increasing doses of 25, 100 and 400 nmol CPT inhibit tumor yield by ~33, 78 and 96% respectively (Figure 4A) and the tumor weight/mouse by 28, 89 and 98% (Table II, groups 8–10). However, the lower dose of CPT tested neither delayed nor inhibited the incidence of skin tumors promoted by TPA (Figure 4B).

The two-stage tumor promotion protocol yields fewer tumors than the complete promotion process (1–3). Only four applications of TPA in stage 1 were insufficient to produce any tumors in initiated mice (1–3, 9). As already demonstrated in SENCAR and CF-1 mice (1, 9, 17, 18), MEZ alone applied twice a week in the absence of stage 1 is a weak complete tumor promoter, producing ~35% of mice with tumors and an average of 1 tumor/mouse (Figure 5). As shown before (1, 9, 17, 18), TPA in the first stage clearly enhances the tumor-promoting activity of MEZ (Figure 5). For instance, there was a 7-fold increase in the average tumor weight/mouse when MEZ treatments were applied following stage 1 by TPA (Table III, group 2 versus 1) and that is why it is concluded that MEZ is a weak complete tumor promoter but a good second stage tumor promoter. When administered 12 h after each application of TPA in the first stage 400 nmol CPT inhibited the final incidence, yield and weight of skin tumors at 20 weeks by ~35, 56 and 39% respectively (Figure 5 and Table III, group 3). The same CPT post-treatment given after each application of MEZ in stage 2 inhibited the final incidence, yield and weight of skin tumors at 20 weeks by ~50, 78 and 84% respectively (Figure 5 and Table III, group 4). Whether or not they are treated twice a week with 400 nmol CPT during stage 1, stage 2 or complete tumor promotion, SENCAR mice gain body weight and survive at the same rate (Tables II and III), indicating that these chronic topical applications to the skin of Topo I inhibitors are not toxic.
Table III. Inhibitory effects of CPT post-treatments in the two-stage tumor promotion protocola

<table>
<thead>
<tr>
<th>Group</th>
<th>Two-stage tumor promotion</th>
<th>Week of 1st Pa</th>
<th>Observations at week 20</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Stage 1 by TPA (4X/2 weeks at time 0)</td>
<td>Stage 2 by MEZ (36X/18 weeks at time 0)</td>
<td>Mouse wt (g)</td>
</tr>
<tr>
<td>1</td>
<td>TPA</td>
<td>MEZ</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>TPA + CPT (400 nmol, +12 h)</td>
<td>MEZ</td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td>TPA + CPT (400 nmol, +12 h)</td>
<td>MEZ</td>
<td>6</td>
</tr>
<tr>
<td>4</td>
<td>TPA</td>
<td>+ CPT</td>
<td>9</td>
</tr>
</tbody>
</table>

aThe conditions of the tumor experiment are detailed in Figure 5.
bExpressed as a percentage of group 2.

Discussion

Topo I is an intranuclear enzyme that relaxes supercoiled DNA by creating and resealing single-strand breaks (SSBs) (19,20). However, CPT stabilizes the reversible covalent Topo I-DNA intermediate and forms a covalent ternary complex Topo I-CPT-DNA, which prevents religation of the SSB. CPT is a S phase-specific anticancer drug because collision between the advancing replication fork and the ternary complex creates highly toxic and long-lived DNA double-strand breaks (DSBs) (21-24). Relative to normal tissues, the increased levels of Topo I observed in several types of tumors, including skin melanomas, suggest a therapeutic advantage for cancer treatment with Topo I-targeting drugs (25,26).

The desired and undesired effects of CPT on specific stages of tumorigenesis are difficult to predict. CPT does not prevent formation of covalent DMBA-DNA adducts in vivo (7), suggesting that formation of cleavable complexes by CPT neither alters the metabolic activation of the procarcinogen nor interferes with covalent binding of the ultimate carcinogen to DNA, two events required for tumor initiation and complete carcinogenesis (2,3). Under the present experimental conditions CPT pre- and post-treatments inhibited the yield, but not the incidence, of skin tumors initiated by DMBA. The mechanism of tumor promoter-induced enhancement of tumor initiation suggests that epidermal cells synthesizing DNA may be more susceptible to initiation by certain carcinogens (14-16). DNA replication may play a crucial role in tumor initiation by irreversibly 'fixing' the initiating genetic damage before it can be repaired. Since the covalent binding of [%H]DMBA to epidermal DNA peaks at 24 h (27), DNA replication might be a more effective enhancer of tumor initiation if it is stimulated by TPA after maximal interaction of DMBA with DNA (14-16). Taken together, the partial inhibitory effects of CPT obtained under the present experimental conditions (Figures 1 and 2 and Table II, groups 3-5) suggest that, even though CPT cannot prevent covalent binding of the initiator to its DNA target, the Topo I inhibitor may indirectly limit the tumor-initiating activity of DMBA because it decreases the basal or TPA-stimulated levels of epidermal DNA replication during DMBA initiation, enhances even further the early inhibition of DNA synthesis caused by this initiator (Table I) and, consequently, may reduce the level of initiating mutations that are fixed in epidermal stem cells. If basal and TPA-stimulated DNA replication have a greater impact on tumor initiation after rather than before the peak of covalent binding of DMBA to DNA, it is speculated that CPT might be a more

Fig. 4. Dose-dependent inhibition of skin tumor promotion by CPT post-treatments. The experiment was as described in Figure 1. The dose of 400 nmol CPT was dissolved in 0.4 ml DMSO:acetone (1:9) and this solution was diluted 1/4 with acetone to administer the lower doses tested. Vehicle (△) or 25 (○), 100 (●) or 400 nmol CPT (△) were applied 12 h after each promotion treatment with 3.24 nmol TPA.
Fig. 5. Inhibitory effects of CPT in the two-stage tumor promotion protocol. All mice were initiated with a single dose of 20 nmol DMBA. One week later all mice were promoted twice a week for 2 weeks with 3.24 nmol TPA to achieve stage 1 and then twice a week for 18 weeks with 3.05 nmol MEZ to achieve stage 2 (●). The dose of 400 nmol CPT was applied 12 h after either each TPA treatment in stage 1 (○) or each MEZ treatment in stage 2 ( □). Initiated mice received only 36 applications of MEZ during stage 2 to assess the weak complete tumor-promoting activity of this agent in the absence of stage 1 treatment with TPA ( □).

A single dose of 500 nmol CPT applied 7 h after TPA can inhibit the entire time course for stimulation of DNA synthesis observed 16–64 h after tumor promoter application (7), but a dose of CPT 20 times smaller may have a more limited duration of action. The 12 h post-treatment with 25 nmol CPT, therefore, might inhibit TPA-stimulated DNA synthesis at 16 h by ~50% (7), but lose its effectiveness thereafter (Figure 4 and Table II, group 8). A closer analysis of Figure 5 and Table III reveals that, in fact, CPT totally inhibits the stage 2 tumor-promoting effects of MEZ, since in the presence of the Topo I inhibitor (group 4) the tumor-promoting activity of the sequential combination TPA (stage 1) and MEZ (stage 2) (group 2) is the same as the basal level of tumor promotion by MEZ alone (group 1). The apparently greater antitumor-promoting effect of CPT in stage 2 may simply be due to the fact that stage 1 represents only 10% of the 20 week tumor promotion process and the drug is applied 36 times in stage 2, but only 4 times in stage 1.

Although in vitro analyses do not necessarily reflect chemo-
therapeutic efficacy, the potency of the drugs in inhibiting Topo I catalytic activity is generally correlated with their differential antitumor activity (4,5). TPA transiently increases Topo I mRNA levels in human skin fibroblasts and Topo I activity is increased by phosphorylation with protein kinase C (PKC) in vitro (37,38). Phosphorylation of Topo I occurs rapidly in TPA-treated HL-60 cells, suggesting that PKC controls Topo I phosphorylation in vivo (38). Once the signal for PKC activation is transduced, continuous exposure to TPA is not necessary for the maximum effect on Topo I expression. TPA may affect Topo I gene expression at the transcriptional level (37). The Topo I gene can be categorized as an immediate early response gene to TPA stimulation (37). Phosphorylation may be a physiological means of activating or localizing Topo I for transcription during the mitogenic response of mammalian cells (39). Because overexpression of Topo I may render cells more sensitive to the effects of CPT, the parent drug and its derivatives might be particularly effective against Topo I catalytic activity, DNA synthesis and clonal expansion of tumor cells if TPA- and MEZ-treated epidermises were to contain high concentrations of their target enzyme.

In this study CPT inhibited the promoting much more than the initiating component of carcinogenesis, but the specific anti- and, eventually, pro-carcinogenic effects of this Topo I inhibitor should be further characterized under different experimental conditions. Even though they do not prevent the initiator from interacting with DNA, CPT post-treatments might effectively inhibit tumor initiation if they are applied at times when they can block the replication of DNA required to fix the initiating mutations. CPT may be the first drug to be demonstrated to inhibit tumor promotion when applied 1 or 2 days after TPA. The ability of CPT to affect the growth and malignant progression of pre-existing PAs remains to be evaluated. This study suggests that the model of multistage carcinogenesis in mouse skin may be valuable to determine if new optically pure CPT analogs are more effective than their derivatives. Further characterization of skin tumor promotion and progression by experiments in SENCAR mice. Cancer Res., 36, 773-780.

Acknowledgements

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References


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