Inhibition of cellular transformation by triphenylmethane: a novel chemopreventive agent

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Triphenylmethane (TPM) was found to inhibit 3-methylcholanthrene-induced neoplastic transformation of 10T1/2 cells in a dose-dependent manner (ED₅₀ = 2.8 μM). This activity was independent of any effect on intercellular communication and did not appear to be directly related to the general antioxidant properties of TPM as measured by cellular thiobarbituric acid-reactive substances. Triphenylmethanol (TPMOL) and diphenylmethane also inhibited transformation (ED₅₀ = 6.9 and 90 μM respectively). TPM had no effect on the proliferation of exponentially growing cells. At higher concentrations TPM and its analogues enhanced plating efficiency of cells indicating no significant toxicity for these compounds at levels up to 50 μM. The inhibitory effects of TPM on transformation were reversible when TPM was removed from the medium. While TPM had no effect on the growth of fully transformed cell lines, it was able to inhibit the growth of 1/3 neoplastic foci in the presence (but not absence) of 10T1/2 cells. TPM was found to stimulate protein kinase C (PKC) activity for both crude C3H10T1/2 cytosolic PKC and purified PKC obtained from rat brain. The ability of TPM to stimulate PKC activity appeared to be dependent on [CaCl₂] and the order of reagent addition in the assay. Tamoxifen, a structurally related compound to TPM, was also found to enhance PKC activity over the same concentration range but was less potent than TPM. The biological effects of TPM and related compounds indicate that they function in a manner distinct from other highly unsaturated transformation inhibitors such as carotenoids and retinoids. The inability of triphenylene to inhibit transformation suggests that a reactive methyl carbon may be essential for activity.

Introduction

While considerable research has been devoted to the therapeutic treatment of malignancies, it is only recently that interest has begun to focus on the prevention of cancer in the general population (1,2). Prevention strategies currently are focused in two primary areas: (i) the reduction of cancer incidence rates through dietary and lifestyle interventions, directed towards two primary areas: (i) the reduction of cancer incidence rates (1,2); (ii) the use of cancer chemopreventive agents in individuals at high risk for cancer such as the use of tamoxifen in breast cancer. The most widely studied chemopreventive agents are the retinoids which have been demonstrated to decrease the incidence of neoplasia at several anatomic sites (3,4) including head and neck (5). The high levels of provitamin A carotenoids contained in a risk-reducing diet may well contribute to this activity (6).

Extensive studies have shown that natural and synthetic retinoids reversibly inhibit 10T1/2 cell transformation at non-toxic doses during the post-initiation phase of carcinogenesis (7). Diverse carotenoids have comparable effects (8,9) although they are less potent on a molar basis than retinoids (10). For both classes of molecule, chemopreventive activity is directly correlated with the induction of gap junctional intercellular communication which in both cases results from enhanced expression of connexin 43 protein (Cx43*) (11,12), a member of a family of transmembrane junctional proteins. Carotenoid activity appears independent of the provitamin A status of the individual molecule, as canthaxanthin, and to a lesser extent lycopene, both have activity, yet neither acts as a source of retinoids in mammals (13). Neither does activity correlate with a measure of their ability to act as lipid-phase antioxidants. Furthermore α-tocopherol, an extremely potent antioxidant, exhibits only moderate chemopreventive action in 10T1/2 cells (9) and does not up-regulate Cx43 gene expression (L.-X. Zhang, R.V. Cooney and J.S. Bertram, submitted). The 10T1/2 cell transformation assay has been a valuable tool for assessing the ability of various chemical agents to inhibit the transformation process and for mechanistic studies of their action (14). Dietary antioxidants such as vitamin C (15), vitamin E (16) and protease inhibitors (17), which have been inversely associated with cancer incidence for a variety of sites in humans, have also been shown to block transformation in 10T1/2 cells (9,18,19).

Chemoprevention clinical trials involving the use of many different chemopreventive agents have recently been started (20–22) despite an often incomplete understanding of the molecular biological properties of these compounds. Previous work in our laboratories has investigated the relationship between carotenoid structure and anti-transformation activity (8,9,12). A characteristic of many active carotenoids is the possession of a conjugated polyene system (11 conjugated double bonds in the case of β-carotene). As part of our ongoing study of the mechanism of action of diverse carotenoids we observed that triphenylmethane (TPM), a highly unsaturated, non-carotenoid model compound exhibited potent inhibitory activity in the 10T1/2 transformation assay. Watanabe et al. (23) reported that TPM (350 mg/kg i.p. for 3 days) possessed anti-neoplastic properties in vivo and increased the survival time of mice with experimentally induced Ehrlich ascites carcinoma. No toxic effects for TPM or triphenylmethanol (TPMOL) were observed when given i.p. at 500 mg/kg in those studies. In an effort to define the nature of these actions better, this paper describes the effects of TPM and structurally related compounds (Figure 1) on chemically-induced transformation and details our attempts to understand the mechanistic basis for this activity.
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of confluent cultures as described previously (10). Heterologous communication
using gap junctional communication assay (10) was measured according to the method described by Hossain et al. (27), modified as described above. The samples consisted of 250 µM histidine H1, 26 µM ATP, 0.6 µCi (32P)ATP, 5 mM MgCl₂, 75 mM NaCl, 20 mM Tris pH 7.4, 1 mM EDTA, 0-3.5 mM CaCl₂, 250 mM histone HI, 26 µM ATP, 0.6 µCi (32P)ATP, 5 mM MgCl₂, 75 mM NaCl, 20 mM Tris pH 7.4, 1 mM EDTA, 0-3.5 mM CaCl₂. The samples were vortexed, incubated for 8 min at 30°C in a shaking water bath and transferred to filter paper squares.

Gap junctional communication assay

determined by the microinjection of a fluorescent dye (10% Lucifer yellow CH in 0.33 M LiCl) at constant pressure into cells of confluent cultures as described previously (10). Heterologous communication was measured according to the method described by Hossain et al. (10) using fluorescent microspheres (Polyscience, Warrington, PA) as labels for the transformed cells.

Materials and methods

Chemicals

Highly purified TPM (99.9%), triphenylene (TPE) (98%), TPMOL (97%) and diphenylmethane (DPM) (99%) were obtained from Aldrich Chemical Co., Milwaukee, WI. Purified rat brain protein kinase C (PKC) was purchased from Calbiochemical Corp., La Jolla, CA. Phosphatidyl serine was obtained from Avanti Polar-Lipids, Inc. Pelham, AL. Tamoxifen, staurosporine, ATP, histone H1 and buffers were purchased from Sigma Chemical Co., St. Louis, MO. [32P]ATP was from ICN Radiochemicals/Biochemicals, Irvine, CA.

Cell culture and transformation assay

C3H/10T1/2 mouse embryo fibroblasts were cultured in basal Eagle’s medium (BME) supplemented with 5% calf serum (Hyclone) containing 25 µg/ml gentamicin. In experiments designed to measure possible cytotoxicity, 10T1/2 cells were plated at 200 cells per 60 mm dish and treated 24 h later with TPM, TPMOL, TPE or DPM delivered in acetone (final concentration of acetone = 0.5%). After 10 days cell colonies were fixed with methanol, stained with Giemsa and colony numbers scored as plating efficiency (PE). The protocol for the transformation assay was as described previously (8,9). Treatment with TPM or related compounds were begun 7 days after removal of MCA at the time of feeding and continued on a weekly basis for the duration of the assay.

To measure the effect of TPM on the log-phase growth, C3H/10T1/2 cells or transformed clone 4B cells were plated in 60 plate cultures at a seeding density of 10⁶ cells in medium containing either 0.5% acetone, 3 × 10⁻⁶ M TPM or 3 × 10⁻³ M TPM. The mean number of cells/dish for four determinations of cell number were measured for each concentration over a period of 8 days.

PKC assay

Preparation of cell cytosol. 10T1/2 cells were plated on 10 cm tissue culture plates in media containing 5% calf serum with weekly refeeding. Cells from three or four plates were harvested at 4 days (nearly confluent), 7 days (confluent) or 11 days. Cells were homogenized and fractionated according to the method described by Hill et al. (28). Supernatants were pooled to a volume of 4 ml with a protein concentration averaging 0.5 mg/ml. Cytosols were assayed on the same day as extraction.

Purified rat brain PKC. The enzyme was diluted into extraction buffer to which 10 mM mercaptoethanol, 0.7% glycerol and 26.8 µg BSA were added (25).

Enzyme assays. PKC activity was assayed in triplicate (25,26), with PKC activity defined as the difference in acid-precipitable c.p.m. between samples incubated in the presence or absence of phosphatidyl serine vesicles expressed as pmol phosphate/min. Reagents were added in the following order: 40 µl of cytosolic extract was added to 10 µl containing 20 µg of phosphatidyl serine vesicles. PKC effectors were added in 1 µl volume; TPM or tamoxifen in acetone; staurosporine, 10.7 µg in 1 µl H₂O. After mixing, tubes were preincubated on ice for 10 min to ensure binding of these components (25). The reaction was started by the addition of 30 µl of the remaining phosphorylating solution containing the substrate and cofactors (final concentrations in the 80 µl reaction mixture were: 25.0 µg histidine H1, 26 µM ATP, 0.6 µCi (32P)ATP, 5 mM MgCl₂, 75 mM NaCl, 20 mM Triath pH 7.4, 1 mM EDTA, 0-3.5 mM CaCl₂). The samples were vortexed, incubated for 8 min at 30°C in a shaking water bath and transferred to filter paper squares.

Lipid peroxide assay

Cellular lipid peroxide levels were assessed by measuring the amount of thiobarbituric acid (TBA)-reactive material in 5 x 10⁶ cells according to the method of Yagi (27). Cells were cultured in 60 dishes as described above. Triphenylmethane or other compounds were added in acetone (final acetone concentration = 0.5%) at the time of media change. Medium was removed from a dish of confluent 10T1/2 cells and the cells were rinsed twice with 2.5 ml portions of phosphate-buffered saline (PBS). The cells were scraped from the dish with a rubber policeman into 5.0 ml of PBS, centrifuged at 3000 r.p.m. for 10 min and the supernatant removed. The cell pellet was then thoroughly mixed with 2.0 ml of 0.083 N H₄SO₄. To this mixture, 0.25 ml of 10% phosphotungstic acid was added and mixed and the sample allowed to stand for 5 min at room temperature. The mixture was then centrifuged at 3000 r.p.m. for 10 min and the supernatant discarded. The pellet was mixed with 1.0 ml of 0.083 N H₂SO₄ and 0.15 ml of 10% phosphotungstic acid, then centrifuged at 3000 r.p.m. for 10 min. The pellet was suspended in 2.0 ml of distilled water and 0.5 ml of TBA reagent (equal volumes of aqueous 0.67% TBA and glacial acetic acid) and heated at 95°C for 1 h. After cooling, 2.5 ml of n-butanol was added, the mixture vortexed vigorously and centrifuged for 5 min at 3000 r.p.m. The butanol layer was removed for spectrophotometric analysis as described previously (27).

Results

TPM blocked the formation of foci in transformation assays in a dose-dependent manner with an ED₅₀ = 2.8 µM (Figure 2). TPMOL, lacking the benzylic hydrogen of TPM, also exhibited potent inhibitory activity over a similar concentration range (ED₅₀ = 6.9 µM), while DPM was approximately an order of magnitude less effective than TPM or TPMOL. Figure 2). TPE had no significant effect on transformation when tested up to 10⁻⁴ M (data not shown). Cessation of TPM treatment resulted in the reversal of inhibition of transformation as evidenced by the appearance of foci in the previously treated dishes after 13 days in the absence of TPM (Table I). Three cell lines were cloned from three separate Type III foci, one of which (TPMII) was found to be growth inhibited when plated on a confluent monolayer of 10T1/2 cells in the presence of, but not in the absence of 3 x 10⁻⁵ M TPM (Table II). This particular clone was non-tumorigenic in nude mice, however, after repeated passage in cell culture it lost its responsiveness to TPM and behaved like other fully transformed cell lines. Two clones isolated from the MCA-treated controls were found to be tumorigenic in nude mice. Other fully transformed cell lines showed little or no response to TPM treatment (Table II).

DPM had no effect on the log-phase growth of 10T1/2 cells or the transformed cell line clone 4B (data not shown). Concentrations of TPM, TPMOL, DPM or TPE up to 5 x 10⁻⁵ M were well tolerated and showed a consistent positive effect on the plating efficiency of 10T1/2 cells (Figure 3).

In previous studies we have shown that reversible suppression of chemically-induced transformation in 10T1/2 cells is caused by agents which up-regulate gap junctional intercellular communication (10). Carotenoids and retinoids increase homologous communication and prevent transformation of initiated cells (10,12), while agents such as RO20-1724, which elevate CaM levels increase heterologous (i.e. normal:transformed) communication and prevent the growth of transformed cells when in contact with normal cells (14). To determine if TPM and related molecules exert their actions via similar mechanisms, their influence on homologous (10T1/2:10T1/2) and heterologous communication was measured (Figure 4). Homologous communication was significantly inhibited by TPM after 7 days treatment as compared to acetone-treated controls, however, communication measured at 12 days showed no significant difference. No significant effect on intercellular communication was observed between C3H10T1/2 cells and the neoplastic cloned cell line 4B (control levels of intercellular communication were extremely
Fig. 2. Inhibition of transformation by TPM, TPMOL and DPM. Post-initiated cells were treated with TPM (black) or its analogues in acetone (final acetone concentration = 0.5%) weekly at the time of media change for a period of 4 weeks as described in the text for the transformation assay. The values reported for TPM represent the means of 24 dishes from two separate experiments. Control MCA-treated dishes contained an average of 1.83 foci/dish (n = 48) for a mean transformation frequency (TF) of 1.01 ± 0.32. Negative control dishes contained 0 foci (n = 12). Values represent means of multiple determinations from three separate experiments for TPMOL (O) in which the average no. of foci/dish for positive controls = 1.14 (n = 69) for a mean TF of 0.84 ± 0.26. Values reported for DPM (A) are the means of two separate experiments (values without error bars were from a single experiment, n = 12 for each concentration). Mean TF for positive controls = 0.59 ± 0.02 (n = 60) while the average no. of foci/dish = 0.93

Table I. Reversibility of TPM inhibition of transformation

<table>
<thead>
<tr>
<th>Carcinogen treatment</th>
<th>TPM [M]</th>
<th>Fixation time</th>
<th>Dishes</th>
<th>Dishes with foci</th>
<th>Foci/dish</th>
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<td>2</td>
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<tr>
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<td>4</td>
<td>0.33</td>
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<tr>
<td>MCA 10^{-5a}</td>
<td>-</td>
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<td>12</td>
<td>11</td>
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<tr>
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<td>12</td>
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<tr>
<td>MCA 3 x 10^{-5a}</td>
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<td>43</td>
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Cultures were treated with MCA, then with the indicated concentration of TPM for 4 weeks, as described in the legend to Figure 2. Cultures were then divided; half were fixed and stained while the remainder were maintained drug-free for an additional 13 days. These were then fixed, stained and the transformed foci counted.

low so a statistically significant decrease in communication is difficult to detect), while heterologous communication between 10T1/2 cells and clone TPMM, described above, was inhibited by as little as 3 x 10^{-5} M TPM (P < 0.01). In addition, TPM treatment did not significantly increase cAMP in 10T1/2 cells above control levels (data not shown). As a positive control RO20-1724 produced the expected increase in heterologous communication and elevation of cAMP levels. It may be concluded that TPM does not exert its effects via modulation of junctional communication.

Lipid peroxide levels in 10T1/2 cells exposed to various concentrations of TPM, TPMOL, TPE, DPM or α-tocopherol were determined by measuring the amount of TBA-reactive material in cell membranes by adapting the method of Yagi (27). Cells were assayed in duplicate at six intervals over a 2 week period after treatment. Figure 5 shows the mean levels of TBA-reactive material in cell membranes by adapting the method of Yagi (27). Figure 5 shows the mean levels of TBA-reactive material in cell membranes by adapting the method of Yagi (27). Figure 5 shows the mean levels of TBA-reactive material in cell membranes by adapting the method of Yagi (27).
Fig. 4. Effect of TPM on junctional communication. Homologous intercellular junctional communication was measured in 10T1/2 cells (A) after either 7 days (open bars) or 12 days (hatched bars) of TPM treatment as described in Materials and methods. Heterologous communication (B) was determined for clone TPM_1 cells or clone 4B cells labelled with fluorescent beads and seeded on a lawn of confluent C3H10T1/2 cells. The extent of dye transfer between heterologous cell types was determined as described in Materials and methods. Each value represents the mean number of communicating cells ± SD for a minimum of 10 injected cells. RO20 represents RO20-1724, a potent inhibitor of cAMP phosphodiesterase shown previously to enhance heterologous junctional communication (14). * P < 0.05, **P < 0.01.

Fig. 5. Effect of TPM analogues on lipid peroxidation in C3H10T1/2 cells. Cells were assayed for TBA-reactive substances, as described in Materials and methods, six times over a 2 week period (two dishes each at days 2, 5, 7, 9, 12 and 14 post-treatment). TPM (or analogue) was administered at the time of media change (days 1 and 8). The data for the six time points were averaged and each value represents the mean of 10 dishes ± SD (the high and low value for each set were disregarded). Acetone controls and treated dishes all contained 0.5% acetone final concentration. Concentrations for test substances were 5 x 10^{-6} M, 10^{-5} M, 10^{-4} M, 10^{-3} M, 10^{-2} M, and 10^{-1} M. *P < 0.05 for the difference in means relative to the control.

The effect of TPM on 10T1/2 PKC activity was determined for cells at near-confluency, 4 days after plating (Figure 6). Cells were also assayed at 7 and 11 days after plating with essentially similar results (data not shown). For comparison, the assay was also carried out simultaneously using identical concentrations of tamoxifen. Staurosporine (10.7 pmol/tube) was used as a control inhibitor. Regardless of cell age, optimal concentrations of TPM enhanced PKC activity ~2-fold over control levels, while tamoxifen exhibited a similar but more modest effect and became inhibitory at high concentrations. The concentration at which a maximal effect was observed varied slightly with the age of the cells, older cells requiring slightly higher levels of TPM for PKC activation. Staurosporine strongly inhibited PKC activity (83-100% inhibition) as expected. The ability of TPM to activate PKC appeared to depend strongly on the CaCl_2 concentration for both C3H/10T1/2 cells and for the purified enzyme from rat brain (Figure 7).
Discussion

TPM is able to inhibit transformation at concentrations comparable to those used in carotenoid studies \(ED_{50}\) for active carotenoids is \(\sim 10^{-6}\) M (9) and approximately an order of magnitude higher than that required for retinoids such as retinol (10). However, the activity of TPM appears mechanistically unrelated to either of these classes of compounds since TPM does not enhance intercellular communication as do carotenoids and retinoids (10,12). In contrast the data show that in some instances TPM inhibits communication, which is consistent with the observed effect of TPM on PKC activity. Some of the biochemical properties of TPM more closely resemble those of the tumor-promoting phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA), which also activates PKC by enhancing the affinity of the enzyme for \(Ca^{++}\) (26) and inhibits intercellular communication (12), yet acts as a promoter of transformation (29) in stark contrast to TPM. Recently, however, PKC activation by TPA has been reported to cause differentiation and inhibition of proliferation in U-937 leukaemia cells (30). Tamoxifen is reported to compete with TPA for binding to membrane receptors (31), while okadaic acid, a known tumor promoter in skin, also inhibits transformation in 10T1/2 cells (32), possibly through the down-regulation of growth factor receptors (33).

It is unlikely that TPM is blocking transformation by acting as a general growth inhibitor as we could find no significant effects on the growth of C3H10T1/2 cells or transformed cell lines at the concentrations used in this study. The enhanced plating efficiency observed in the presence of TPM also demonstrates that TPM and its analogues are non-toxic. Interestingly, the observed effects of TPM on plating efficiency correlate well in concentration and magnitude with the \(in\ \text{vitro}\) effects of TPM on PKC activity. Further, the inhibitory effects of TPM on the growth of clone TPM6 when cultured on 10T1/2 cells was not due to any direct inhibition of growth of this particular clone as growth was not inhibited in the absence of 10T1/2 cells (Table II).

In light of the rather unique and contradictory properties of TPM, it is possible that TPM is inhibiting the development of transformed foci, not by acting upon the initiated cell directly, but rather, is causing the balance of normal 10T1/2 cells to be more resistant to invasion and overgrowth by preneoplastic, initiated cells, perhaps by stimulating the growth of normal cells or the intercellular matrix. Previously it was reported that ascorbate could also block the growth of \(\sim 40\%\) of cloned foci in replating experiments similar to those described here (18), however, this was apparently unrelated to any effect on the collagen matrix. Recently, staurosporine has been reported to inhibit invasion of malignant cells by inhibiting PKC (34,35). Tamoxifen is believed to act as a chemopreventive agent by blocking the oestrogen-mediated stimulation of proliferation of breast tissue through competition for the oestrogen receptor, however tamoxifen is also thought to have more general, but less well defined, chemopreventive effects as well. It has been reported that tamoxifen can act as an inhibitor of PKC in rat brain (31,36) and may thereby block signal transduction pathways leading to cell division and/or invasion. Although we do observe inhibition of PKC at high concentrations in agreement with these studies, the enhancement of PKC activity at lower concentrations of tamoxifen, as we observed, was not previously reported. Others have concluded that tamoxifen analogues block binding of ATP to the catalytic site of the molecule (25) and may inhibit PKC in this manner. The order of addition in the assay appears to be critical in our experiments, as no enhancing effect of TPM was observed if the TPM was added to the phosphatidylyserine vesicles prior to the enzyme or if CaCl\(_2\) was reduced. The enhancing effect of TPM on PKC activity may be due to a membrane effect resulting from the introduction of highly unsaturated molecules into the lipid bilayer (37).

Tamoxifen has also been reported to inhibit lipid peroxidation and the biologically more active analogue 4-hydroxytamoxifen is correspondingly more potent at inhibiting lipid peroxidation (38). Although TPM and its analogues did reduce cellular lipid peroxide levels, it seems unlikely that the general antioxidant properties of TPM as measured by the TBA assay are sufficient to explain the observed effects on transformation, as DPM was equally effective in preventing peroxide formation but was significantly less effective in blocking transformation. In addition, \(\alpha\)-tocopherol was much more active as an antioxidant, yet at \(10^{-6}\) M only blocks transformation by 65\% (9). The complete absence of anti-transformation activity by TPE as well as the similarity between the relative \(ED_{50}\) and the ease of formation of the triphenylmethyl radical from TPM and TPMOL (39), suggest that the generation of the triphenylmethyl radical may somehow play a role in the biological activity of these compounds either as an antioxidant or as a reactive intermediate, perhaps in an electron transfer reaction. Such a role for stable aromatic radical intermediates in biological systems is not without precedence (40). Triphenylmethyl compounds have been reported to have a variety of biological effects including anti-mitotic and neurological properties and most of these have been associated with the active carbon centre of the methyl group (41). The possible role of TPM as an electron donor may be a reasonable explanation for some of its biological activities. In this respect there are similarities to ascorbate which can also act as an electron donor and has analogous effects on the transformation of 10T1/2 cells (18), including the ability to block the growth of some cloned foci on normal monolayers of 10T1/2 cells.

TPM represents a novel class of compounds capable of inhibiting the promotional phase of transformation while causing cellular changes normally characteristic of tumor promoters, such as PKC activation and inhibition of gap junctional communication. Its mechanism of action is unknown but appears distinct from other chemopreventive agents such as the carotenoids and retinoids. Future advances in the prevention and treatment of neoplastic diseases will require a fundamental understanding of the biochemistry and mechanisms of the carcinogenic process and correspondingly, various means of blocking these processes. The results described here for TPM suggest that chemopreventive agents may be as diverse in structure and mechanism as the processes that lead to carcinogenesis.

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