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Minimal Role of Enhanced Cell Proliferation in Skin Tumor Promotion by Mirex: A Nonphorbol Ester-Type Promoter

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Mirex, a chlorinated hydrocarbon previously used as a systemic insecticide and flame retardant, is a nongenotoxic hepatocarcinogen in both rats and mice. In liver, mirex induced biochemical responses and hyperplasia characteristic of increased cell proliferation, which is consistent with its role as a liver tumor promoter. We have recently shown that mirex is a potent nonphorbol ester-type skin tumor promoter in 7, 12-dimethylbenz[*a*]anthracene (DMBA)-initiated mice. However, unlike its effect in liver, a single topical application of mirex to skin does not induce the acute biochemical responses, such as increased epidermal DNA synthesis and ornithine decarboxylase activity, indicative of increased cell proliferation. Multiple topical applications of mirex over a 1 month period induced only a minimal increase in the number of epidermal nucleated cell layers, which contrasts with definitive hyperplasia induced by a comparable tumor-promoting dose of 12-*O*-tetradecanoylphorbol-13-acetate (TPA). Collectively, these data indicated that mirex is promoting through a novel mechanism. Further evidence that mirex promotes tumors through a mechanism distinct from that of the prototypical skin tumor promoter, TPA, was obtained by examining the effect of their simultaneous co-treatment. The co-application of mirex and TPA yielded a tumor multiplicity greater than the sum of the responses of each promoter individually. In summary, our results demonstrate that mirex, a carcinogenic and hyperplastic agent in liver, is also a very effective tumor promoter in mouse skin, but suggest that mirex operates via a novel mechanism in skin that may involve only a minimal role for enhanced cell proliferation.

Introduction

Recent studies on human colon cancer have suggested that multiple, cumulative genetic lesions are required for carcinogenesis (*1*). However, approximately one-third of the carcinogens detected in the National Toxicology Program rodent bioassay are nongenotoxic in short-term, *in vitro* tests (*2*) and thus may include tumor promoters as defined by experimental models of multistage chemical carcinogenesis. Tumor promoters are carcinogens that have classically been thought to act through nongenotoxic mechanisms to expand populations of cells previously damaged by exposure to a subcarcinogenic dose of a genotoxic initiating chemical (*3*). Because previously identified tumor promoters cause varying degrees of increased cell proliferation, one explanation for their enhancement of carcinogenesis is that these compounds are indirectly genotoxic as a consequence of increasing the occurrence of errors normally made during DNA replication (*4,5*).

Mirex is a chlorinated hydrocarbon previously used as a systemic insecticide and as a flame retardant. Its use in the United States was banned by the Environmental Protection Agency, in part because it is hepatocarcinogenic in both rats and mice (*6–9*). Although it has not been formally tested in an initiation-promotion protocol for liver carcinogenesis, mirex is not genotoxic in short-term, *in vitro* tests (*9,10*) and inhibits gap junctional communication (*11*), two characteristics of tumor promoters. Mirex induces proliferation of hepatocyte smooth endoplasmic reticulum (*12,13*) and mixed-function oxidase activities (*14,15*), as also

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occurs during the adaptive response of liver to other xenobiotic tumor promoters, such as phenobarbital (*16*). Consistent with the above hypothesis relating tumor promotion and cell proliferation, mirex stimulates liver growth, in part through hyperplasia (*17*) which is preceded by induction of ornithine decarboxylase activity (*17,18*) and stimulation of DNA synthesis (*17,19*).

We have recently reported that mirex is a nonphorbol ester-type skin tumor promoter in 7,12-dimethylbenz[*a*]anthracene (DMBA)-initiated female mice (*20*). A maximally promoting dose of mirex (200 nmole, 3 times/week) resulted in a tumor incidence of 96% with a multiplicity of 16 tumors/mouse, comparable to that obtained with thrice weekly applications of 2 nmole of 12-*O*-tetradecanoylphorbol-13-acetate (TPA). However, in contrast to the acute responses of skin to a single application of TPA, mirex did not induce epidermal ornithine decarboxylase activity or increase DNA synthesis. Also, as illustrated here, chronic treatment with 200 nmole of mirex is associated with only a minimal increase in the number of nucleated epidermal cell layers, whereas 2 nmole of TPA causes definitive epidermal hyperplasia. Because these results suggested that mirex promotes through a mechanism distinct from that of TPA, we tested the tumor response to co-application of mirex and TPA. A greater than additive response was observed. Collectively, these results suggest that the mechanism through which mirex promotes skin tumors may involve only a minimal enhancement of cell proliferation. If so, then mirex-induced skin tumor promotion may provide an important exception to the hypothesis linking tumor promotion and epidermal hyperplasia.

Methods

Morphological Examination. Female CD-1 mice (6–8 weeks old) were purchased from Charles River Laboratories (Raleigh, NC). Hair was removed from the dorsal skin with electric clippers. One week later, mice were treated topically three times per week for 4 weeks with 2 nmole TPA or 200 nmole mirex in 200 μL acetone. Forty eight hours after the last application, mice were killed and dorsal skins were excised and fixed for 24 hr in 10% neutral-buffered formalin, were processed routinely, and were then embedded in paraffin. Approximately 5 μm sections were stained with hematoxylin and eosin and viewed for evidence of hyperplasia and toxicity.

Tumor Promotion Experiments. Hair was removed from the dorsal skin of female CD-1 mice (6–8 weeks old) with electric clippers. One week later, mice showing no signs of hair regrowth were initiated with a topical application of 200 nmole 7,12-dimethylbenz[*a*] anthracene (DMBA) in 200 μL acetone. One week after initiation, groups of 30 mice were promoted twice weekly for 20 weeks by topical application of 2 nmole TPA, 200 nmole mirex, or 2 nmole TPA plus 200 nmole mirex. Mice that were promoted with acetone and mice that were initiated with acetone and promoted with mirex or TPA did not develop tumors. One tumor occurred in an acetone-initiated group after 14 weeks of promotion with mirex plus TPA.

Results

We have shown that mirex is a potent skin tumor promoter in DMBA-initiated female CD-1 mice (*20*). After 20 weeks of thrice weekly application, 50 nmole mirex yielded 60% incidence of tumor-bearing mice with an average multiplicity of 4 tumors/mouse, while 200 nmole mirex, maximal promoting dose, yielded 96% tumor incidence with a multiplicity of 16 tumors/mouse. For comparison, thrice weekly application of 2 nmole TPA gave 78% tumor incidence with 15 tumors/mouse. In contrast to the well-characterized biochemical responses to TPA, a single topical application of 200 nmole mirex did not induce epidermal ornithine decarboxylase activity or increase DNA synthesis, as assessed by [^{3}H]thymidine pulse labeling. We have evaluated time courses from 5 to 56 hr for ornithine decarboxylase and from 18 to 108 hr for DNA synthesis to encompass peak response times found with other tumor promoters and have found no evidence of stimulation of these events by mirex (*20*).

As shown in Figure 1, we have morphologically examined skins treated repeatedly (3 times/week for 4 weeks) with comparable promoting doses of mirex (200 nmole) or TPA (2 nmole) for evidence of epidermal hyperplasia. Multiple applications of mirex for 1 month resulted in only a minimal increase in the number of nucleated epidermal cell layers, from one to two in acetone-treated epidermis (Fig. 1*A*) to two to three in mirex-treated epidermis (Fig 1*C*). In contrast, a definitive hyperplastic response of six to seven nucleated epidermal cell layers was observed after repeated treatment with TPA; (Fig. 1*B*). Thus, these results demonstrated that under conditions where both 200 nmole mirex and 2 nmole TPA give similar tumor yields, only the TPA response was associated with the biochemical markers of enhanced cell proliferation, induction of epidermal ornithine decarboxylase activity and increased DNA synthesis, and significant hyperplasia.

These contrasting responses of epidermis to mirex and TPA suggested that distinct mechanisms could be mediating their promotional activities. To test this hypothesis, we determined the tumor response to simultaneous cotreatment with mirex and TPA (Fig. 2). Co-application of 200 nmole mirex with 2 nmole TPA twice weekly for 20 weeks on DMBA-initiated mouse skin yielded a tumor multiplicity of 28 tumors/mouse, compared with 14 tumors/mouse after mirex or 4 tumors/mouse after TPA treatment individually. Rate of tumor incidence was accelerated by cotreatment such that after 8 weeks of promotion, 90% of cotreated mice bore tumors, compared to 47% or 17%

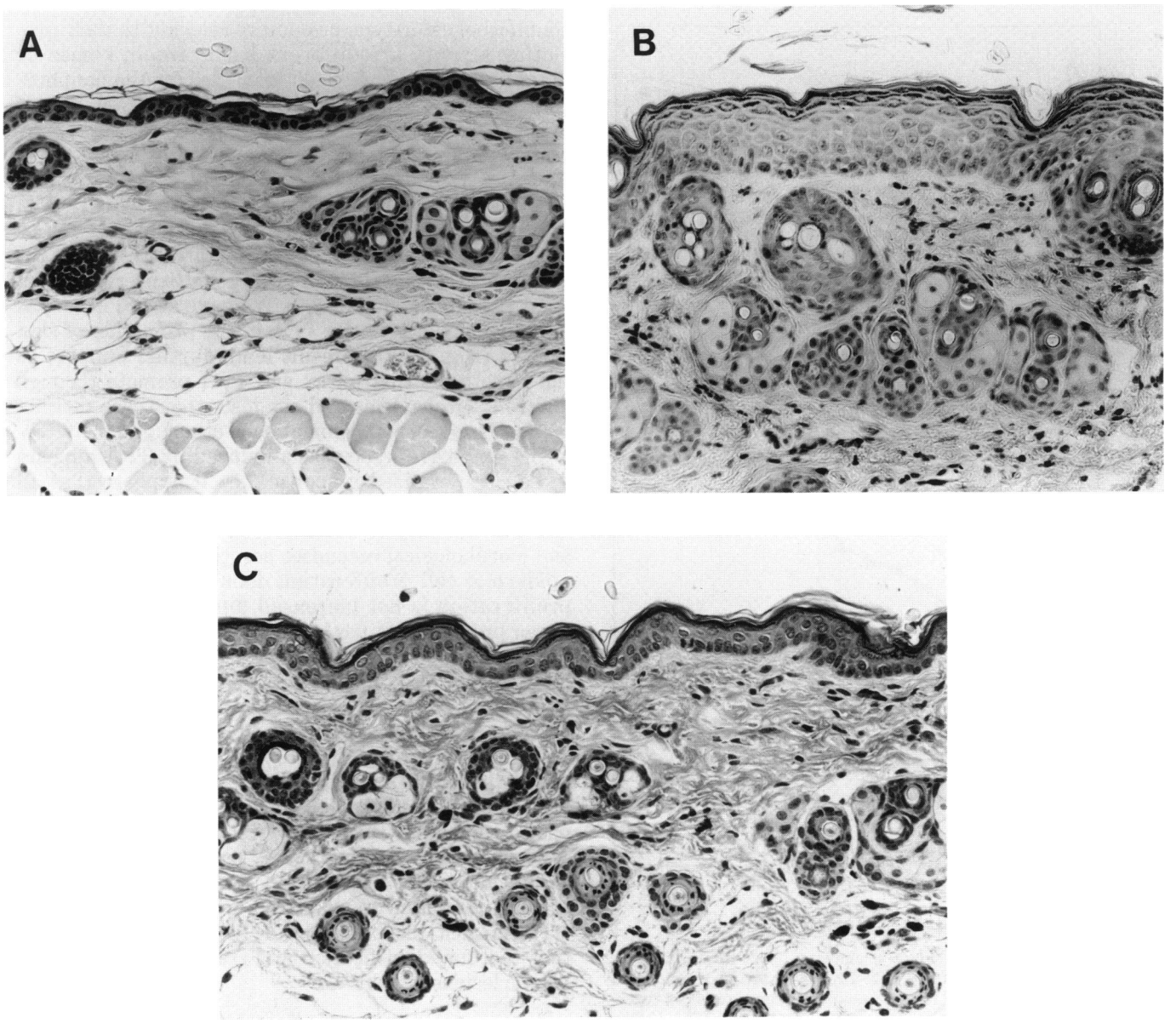

FIGURE 1. Morphological examination of CD-1 mouse epidermis after thrice weekly application for 4 weeks of TPA or mirex. Dorsal skins of mice were treated three times weekly with 200 μL of acetone (*A*), 2 nmole TPA (*B*), or 200 nmole mirex (*C*) in 200 μL of acetone. Mice were killed 48 hr after the last treatment and the skin was removed and processed for light microscopy. Final magnification is 220×.

of mice treated with mirex or TPA individually. Thus, in addition to supporting distinct mirex- and TPA-induced promotional mechanisms, these results further indicate that these two mechanisms could complement each other to yield a synergistic tumor response.

Discussion

We describe here results extending our previous work (*20*) demonstrating that mirex is a potent tumor promoter in DMBA-initiated mouse skin and that mirex promotes skin tumors through a novel mechanism involving minimal cell proliferation. Previously we demonstrated the inability of mirex to induce acute biochemical responses indicative of increased cell proliferation, i.e., increased epidermal DNA synthesis and induction of ornithine decarboxylase activity, that are generally observed in response to single applications of other skin tumor promoters (*20*). Consistent with these results, we have shown that multiple applications of mirex over 1 month did not induce significant epidermal hyperplasia, especially when contrasted with the response to a comparable tumor-promoting dose of TPA (Fig 1). This observation is particularly supportive of a mechanism for mirex-induced tumor promotion that involves a relatively minor role for cell prolifera-

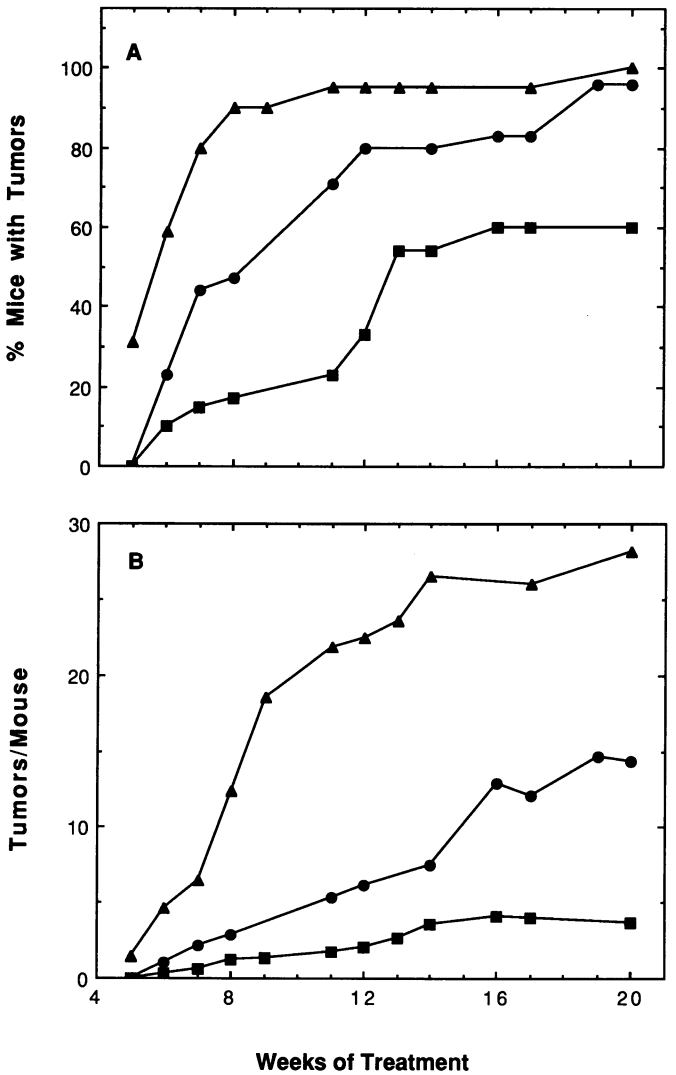

FIGURE 2. Enhanced skin tumor promotion by co-application of mirex and TPA. Groups of 30 CD-1 female mice were initiated with 200 nmole of DMBA, then 1 week later were promoted twice weekly with either 200 nmole mirex (●), 2 nmole TPA (■), or 200 nmole mirex plus 2 nmole TPA (▲). None of the mice initiated with acetone and promoted with mirex or TPA individually or mice initiated with DMBA and promoted with acetone developed tumors. One mouse initiated with acetone and promoted with mirex plus TPA developed one tumor after 14 weeks.

tion, because sustained, chronic hyperplasia has been considered one of the best correlates of skin tumor-promoting activity (*21*).

Skin tumor promoters have been generally divided into two classes, the phorbol ester-type and nonphorbol ester-type, depending on whether they activate protein kinase C (PKC) enzymatic activity or compete with TPA binding to protein kinase C of *in vitro* preparations (*22–24*). We have shown that mirex does not activate brain or epidermal PKC *in vitro* (*25*) and thus is a nonphorbol ester-type promoter. The implication of this classification is that the molecular targets of nonphorbol ester-type promoters relevant to their promoting activity are different from protein kinase C. Potential targets have been identified for the nonphorbol ester-type promoter okadaic acid, an inhibitor of protein phosphatase 1 and 2A (*26*), and for thapsigargin, an inhibitor of the endoplasmic reticulum Ca^{2+}-ATPase (*27*). Further, nonphorbol ester-type and phorbol ester-type tumor promoters can synergize in the induction of common biological responses, such as stimulation of arachidonic acid metabolism in C-9 rat liver cells (*28*) and induction of gene expression from AP-1 sites in Rat-1 cells (*29*). We have shown here that mirex can synergize the TPA in the promotion of skin tumors (Fig. 2). This result lends further support to the hypothesis that mirex and TPA promote through different mechanisms and suggests that these distinct mechanisms can complement each other.

The extensive hyperplasia induced by TPA appears to be a generalized response, i.e., it appears that all basal cells of the epidermis are stimulated to proliferate. Our inability to detect mirex-induced biochemical and morphological responses associated with increased epidermal cell proliferation does not mean that cell proliferation is not increased during mirex-induced tumor promotion. It has been emphasized that the hypothesis relating tumor promotion to cell proliferation predicts that proliferation will be stimulated in initiated cells (*5*). Thus, we speculate that mirex selectively enhances proliferation of initiated epidermal cells without inducing a generalized hyperplastic response. We would thus not expect to see hyperplasia or increased DNA synthesis in uninitiated skin, which is classically used to evaluate these short-term responses. If this speculation is true, then mirex could be an important exception to the correlation that tumor-promoting activity is related to sustained epidermal hyperplasia (*21*). As such, mirex may provide a unique and important tool for identification and study of early preneoplastic lesions in skin.

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REFERENCES

1. Fearon, E. R., and Vogelstein, B. A genetic model for colorectal tumorigenesis. Cell 61: 759–767 (1990).
2. Tennant, R. W., Margolin, B. H., Shelby, M. D., Zeiger, E., Haseman, J. K., Spalding, J., Caspary, W., Resnick, M., Stasiewicz, S., Anderson, B., and Minor, R. Prediction of chemical carcinogenicity in rodents from in vitro genetic toxicity assays. Science 236: 933–941 (1987).
3. Weisberger, J. H., and Williams, G. M. Chemical carcinogens. In: Toxicology: The Basic Science of Poisons, 2nd ed. (J. Doull, C. D. Klassen, and M. O. Amdur, eds.), McMillan, New York, 1980, pp. 84–138.
4. Ames, B. N., and Gold, L. S. Mitogenesis increases mutagenesis. Science 249: 970–971.
5. Cohen, S. M., and Ellwein, L. B. Genetic errors, cell proliferation, and carcinogenesis. Cancer Res. 51: 6493–6505 (1991).

6. Innes, J. R. M., Ulland, B. M., Valerio, M. G., Petrucelli, L., Fishbein, L. Hart, E. R., Pallottla, A. J., Bates, R. R., Falk, H. L., Gart, J. J., Klein, M., Mitchell, I., and Peters, J. Bioassay of pesticides and industrial chemicals for tumorigenicity in mice: a preliminary note. J. Natl. Cancer Inst. 42: 1101–1114 (1969).
7. Ulland, B. M., Page, N. P., Squire, R. A., Weisberger, E. K., and Cypher, R. L. A carcinogenicity assay of mirex in Charles River CD rats. J. Natl. Cancer Inst. 58: 133–140 (1977).
8. Reuber, M. D. Carcinoma and other lesions of the liver in mice ingesting organochlorine pesticides. Clin. Toxicol. 13: 231–256.
9. NTP. Toxicology and Carcinogenesis Studies of Mirex in F344/N Rats. NTP Technical Report Series No. 313, National Toxicology Program, Research Triangle Park, NC, 1990.
10. Maslansky, C. J., and Williams, G. M. Evidence for an epigenetic mode of action in organochlorine pesticide hepatocarcinogenicity: a lack of genotoxicity in rat, mouse, and hamster hepatocytes. J. Toxicol. Environ. Health 8: 121–130 (1981).
11. Trosko, J. E., and Chang, C. Nongenotoxic mechanisms in carcinogenesis: role of inhibited intercellular communication. In: Carcinogen Risk Assessment: New Direction in the Qualitative and Quantitative Aspects. Banbury Report 31, (R. W. Hart and F. D. Hoerger, Eds.), Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 1988, pp. 139–170.
12. Gaines, T. B., and Kimbrough, R. D. Oral toxicity of mirex in adult and suckling rats with notes on the ultrastructure of liver changes. Arch. Environ. Health, 21: 7–14 (1970).
13. Byard, J. L., Koepke, V. C., Abraham, R., Goldberg, L., and Coulston, F. Biochemical changes in the liver of mice fed mirex. Toxicol. Appl. Pharmacol. 33: 70–77 (1975).
14. Baker, R. C., Coons, L. B., Mailman, R. B., and Hodgson, E. Induction of hepatic mixed function oxidases by the insecticide, Mirex. Environ. Res. 5: 418–424 (1972).
15. Mehendale, H. M., Chen, P. R., Fishbein, L., and Matthews, H. B. Effect of mirex on the activities of various rat hepatic mixed-function oxidases. Arch. Environ. Contam. Toxicol. 1: 245–254 (1973).
16. Schulte-Hermann, R. Initiation and promotion in hepatocarcinogenesis. Arch. Toxicol. 60: 179–181 (1987).
17. Yarbrough, J. D., Grimley, J. M., and Karl, P. I. The relationship of ornithine decarboxylase and thymidine kinase to mirex-induced liver growth. Am. J. Physiol. 251: G859–G865 (1986).
18. Mitra, A., Richards, I., Kitchin, K., Conolly, R., and Kulkarni, A. P. Mirex induces ornithine decarboxylase in female rat liver. J. Biochem. Toxicol. 5: 119–124 (1990).
19. Yarbrough, J., Cunningham , M., Yamanaka, H., Thurman, R., and Badr, M. Carbohydrate and oxygen metabolism during hepatocellular proliferation: a study in perfused livers from mirex-treated rats. Hepatology 13: 1229–1234 (1991).
20. Moser, G. J., Meyer, S. A., and Smart, R. C. The chlorinated pesticide mirex is a novel nonphorbol ester-type tumor promoter in mouse skin. Cancer Res. 52: 631–636 (1992).
21. Argyris, T. S. Analysis of the epidermal hyperplasia produced by acetic acid, a poor promoter, in the skin of female mice initiated with DMBA. J. Invest. Dermatol. 80: 430–435 (1983).
22. Fujiki, H., and Sugimura, T. New classes of tumor promoters: teleocidin, aplysiatoxin, and palytoxin. Adv. Cancer Res. 49: 223–264 (1987).
23. Hakii, H., Fujiki, H., Suganuma, M., Nakayasu, M., Tahira, R., Sugimura, T., Scheuer, P. J., and Christensen, S. B. Thapsigargin, a histamine secretagogue, is a non-12-O-tetradecanoylphorbol-13-acetate (TPA) type tumor promoter in two-stage mouse skin carcinogenesis. J. Cancer Res. Clin. Oncol. 111: 177–181 (1986).
24. Suganuma, M., Fujiki, H., Suguri, H., Yoshizawa, S., Hirota, M., Nakayasu, M., Ojika, M., Wakamatsu, K., Yamada, K., and Sugimura, T. Okadaic acid: an additional non-phorbol-12-tetradecanoate-13-acetate-type tumor promoter. Proc. Natl. Acad. Sci. U.S.A. 85: 1768–1771 (1988).
25. Moser, G. J., and Smart, R. C. Hepatic tumor-promoting chlorinated hydrocarbons stimulate protein kinase C activity. Carcinogenesis 10: 851–856 (1989).
26. Haystead, T. A. J., Sim, A. T. R., Carling, D., Honnor, R. C., Tsukitani, T., Cohen, P., and Hardie, D. G. Effects of the tumor promoter okadaic acid on intracellular protein phosphorylation and metabolism. Nature 337: 78–81 (1989).
27. Thastrup, O., Cullen, P. J., Drobak, B. K., Hanley, M. R., and Dawson, A. P. Thapsigargin, a tumor promoter, discharges intracellular Ca^{2+} stores by specific inhibition of the endoplasmic reticulum Ca^{2+}-ATPase. Proc. Natl. Acad. Sci. U.S.A. 87: 2466–2470 (1990).
28. Levine, L., Xiao, D.-M., and Fujiki, H. A combination of palytoxin with 1-oleoyl-2-acetyl-glycerol (OAG) or insulin or interleukin-1 synergistically stimulates arachidonic acid metabolism, but combinations of 12-O-tetradecanoyl-phorbol-13-acetate (TPA)-type tumor promoters with OAG do not. Carcinogenesis 7: 99–103 (1986).
29. Lenormand, P., Muldoon, L. L., Enslen, H., Rodland, K. D., and Magun, B. E. Two tumor promoters, 12-O-tetradecanoylphorbol-13-acetate and thapsigargin, act synergistically via distinct signaling pathways to stimulate gene expression. Cell Growth Differen. 1: 627–635 (1990).