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Overview of Tumor Promotion in Animals

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Our present understanding of two-stage carcinogenesis encompasses almost four decades of research. Evidence for chemical promotion or cocarcinogenesis was first provided by Berenblum, who reported that a regimen of croton oil (weak or noncarcinogenic) applied alternately with small doses of benzo(a)pyrene (BP) to mouse skin induced a larger number of tumors than BP alone. Subsequently, Moltram found that a single subcarcinogenic dose of BP followed by multiple applications of croton oil could induce a large number of skin tumors. These investigations as well as a number of others, such as Boutwell, Van Duuren and Hecker, were responsible in defining many important aspects of the initiation and promotion of two-stage carcinogenesis. The initiation stage in mouse skin requires only a single application of either a direct-acting carcinogen or a procarcinogen and is essentially an irreversible step which as data suggests probably involves a somatic cell mutation. The promotion stage in mouse skin can be accomplished by a wide variety of weak or noncarcinogenic agents and is initially reversible later becoming irreversible. Current information suggests that skin tumor promoters are not mutagenic but bring about a number of important epigenetic changes, such as epidermal hyperplasia, and an increase in polyamines, prostaglandins and dark basal keratinocytes as well as other embryonic conditions. Recently, tumor promotion in mouse skin was shown to consist of at least two stages, in which each stage can be accomplished by either a known promoter or a weak or nonpromoting agent. Some of the important characteristics of the first stage of promotion are: (1) only one application of a first-stage promoter, such as phorbol ester tumor promoters, calcium ionophore A23187, hydrogen peroxide and wounding is needed; (2) the action is partially irreversible; (3) an increase in dark basal keratinocytes and prostaglandins is important; and (4) such an increase can be inhibited by antiinflammatory steroids and protease inhibitors. The second stage of promotion is initially reversible but later becomes irreversible. Polyamines and epidermal cell proliferation are important events in the second stage of promotion. A number of weak or nonpromoting agents, such as mezerein, are effective second-stage promoters which can be counteracted by retinoic acid, antiinflammatory steroids and polyamine synthesis inhibitors. Although skin tumor promotion has been extensively studied in mice, not all strains and stocks of mice are susceptible to phorbol ester tumor promoters. In this regard, the C57BL/6 mice appear to be fairly resistant to phorbol ester tumor promoters. In addition, not all species are equally susceptible to phorbol ester tumor promotion.

Recently the generality of the two-stage system of inducing tumors has been shown to exist in a number of experimental carcinogenesis systems, such as the liver, bladder, lung, colon, esophagus, stomach, mammary gland, pancreas and cells in culture. In these systems, a wide variety of promoting agents such as diet, bile acids, hormones, saccharin, tryptophan, phenobarbital, polychlorinated biphenyls, polybrominated biphenyls and butylated hydroxytoluene have been used to accomplish the tumor promotion stage. It is not presently known if other experimental carcinogenesis systems and the induction of human cancer involves a series of stages similar to that in the mouse skin.

Introduction

Our present understanding of two-stage carcinogenesis encompasses almost four decades of research. Skin carcinogenesis has been known to occur by a two-stage process since Rous and coworkers reported the enhancing effect of irritation on the process of tumor formation. (*1*). Evidence for chemical promotion or cocarcinogenesis was first provided by Berenblum who reported that a regimen of croton oil (weak or noncarcinogenic) applied alternately with small doses of benzo(a)pyrene (BP) to mouse skin induced a larger number of tumors than BP alone (*2*). Subsequently, Mottram (*3*) found that a single subcarcinogenic dose of BP followed by multiple applications of croton oil could induce a large number of skin tumors. These investigators, as well as a number of others, including Boutwell, Van Duuren and Hecker (*4-7*), were responsible for defining many important aspects of the initiation and promotion of two-stage carcinogenesis.

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The characteristics of two-stage carcinogenesis in mouse skin are illustrated in Figure 1. Skin tumors can be induced by the sequential application of a subthreshold dose of a carcinogen (initiation stage) followed by repetitive treatment with a noncarcinogenic promoter (promotion stage). The initiation phase requires only a single application of either a direct or an indirect carcinogen and is essentially an irreversible step, while the promotion phase is initially reversible later becoming irreversible. As shown in Figure 1, a single large dose of a carcinogen such as 7,12-dimethylbenz(a)anthracene (DMBA) is capable of inducing skin tumors in mice. Papillomas occurred after a relatively short latency period (10 to 20 weeks), with carcinomas developing after a much longer period (20-60 weeks). If this dose was lowered as shown in Number 2 of Figure 1, it became necessary to administer DMBA repeatedly in order to induce tumors. If progressively reduced, a subthreshold dose of DMBA was reached which did not give rise to tumors over the lifespan of the mouse. If either croton oil or a phorbol ester such as 12-O-tetradecanoylphorbol-13-acetate (TPA) was subsequently applied repetitively to the backs of mice previously initiated with a single subthreshold dose of DMBA, multiple papillomas appeared after a short latency period, followed by squamous cell carcinomas after a much longer period. The repetitive application of the promoter without initiation by DMBA in general either does not give rise to tumors or produces only a few, and a dose-response relationship is never shown (*8*). If the mice are initiated with a subthreshold dose of a carcinogen such as DMBA, there is an excellent dose response with TPA as the promoter (*8*). Likewise, there is a very good dose response with BP or DMBA as a tumor initiator when the promoter dose is held constant (*8*). Also shown in Figure 1 is the importance of the order of treatments of the initiator and promoter. If repetitive applications of the promoter are administered before initiation, no tumors will develop. The real hallmark of the two-stage carcinogenesis system in mouse skin relates to the irreversibility of tumor initiation. A lapse of up to one year between the application of the initiator and the beginning of the promoter treatment provides a tumor response similar to that observed when the promoter is given only one week following initiation (*4*). Unlike the initiation phase, the promotion stage is reversible, requiring a certain frequency of application in order to induce tumors (*4*). Burns and co-workers have reported results which suggest that there is a progression of certain autonomous papillomas to squamous cell carcinomas, whereas some papillomas are tumor promoter-dependent (*9*). Also (Fig. 1), tumor promotion has been shown to be divided into at least two stages (*10*). The multistage nature of tumor promotion in mouse skin will be described in detail later. Until recently one of the major criticisms of the two-stage carcinogenesis system was its uniqueness to mouse skin and the fact that it was not operational in other tissues and in other species. However, recently the generality of the two-stage system of carcinogenesis has been shown to exist in a number of systems other than the skin such as the liver, lung, bladder, colon, esophagus, mammary gland, stomach, esophagus, pancreas and cells in culture (*11*). Table 1 summarizes the various agents found to have enhancing and/or promoting activity in other organs.

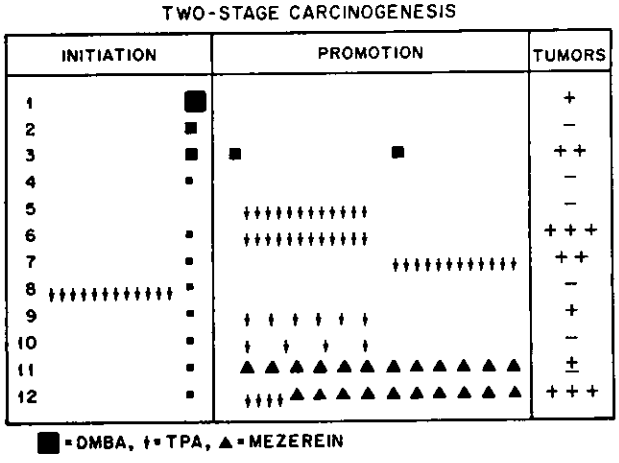

FIGURE 1. Diagram of two-stage carcinogenesis.

Table 1. Tumor promoters and/or enhancing agency for tissues other than the skin.[a]

Organ system	Agent
Liver	Phenobarbital, DDT, BHT, PCB, TCDD, phorbol, thioacetamide, α-hexachlorocyclohexane
Lung	BHT, phorbol
Colon	Bile acids, high fat diet, high cholesterol diet
Bladder	Saccharin, cyclamate, tryptophan
Mammary gland	Hormones, high fat diet, phorbol
Stomach and forestomach	Surfactant, TPA, salt
Esophagus	Diet, alcohol and smoking
Pancreas	Diet, smoking
Mouse cell culture systems	Phorbol esters, saccharin
Rat tracheal organ culture system	Phorbol esters

[a]See Slaga et al. (*11*) for individual references.

Complete and Two-Stage Carcinogenesis in Different Species and Stocks and Strains of Mice

In general, as shown in Table 2, mice are more sensitive to skin carcinogenesis by either the complete carcinogenesis protocol or by the initiation-promotion protocol than rats and hamsters (*11, 12*). The complete carcinogenesis protocol in mice gives rise to a low number of papillomas followed by a high incidence of squamous cell carcinomas, whereas the initiation-promotion protocol gives rise to a large number of papillomas followed by a high incidence of squamous cell carcinomas. Both the complete carcinogenesis and initiation-promotion protocols in rats gives rise to basal cell carcinomas and very few papillomas and squamous cell carcinomas. The complete carcinogenesis protocol in hamsters produces mainly squamous cell carcinomas and some melanomas, whereas the initiation-promotion protocol produces mainly melanomas.

The SENCAR stock of mice was selectively bred for sensitivity to skin tumor induction by DMBA initiation followed by TPA promotion (*12*). Consequently, the SENCAR mouse is extremely sensitive to two-stage carcinogenesis and coincidentally sensitive to complete carcinogenesis (*12*). However, there exist several other stocks and strains of mice that are refractory to promotion or differ in their susceptibility to complete and two-stage carcinogenesis (*12*). Table 3 ranks the susceptibility of several mouse strains and stocks to complete and two-stage carcinogenesis. It is important to emphasize the limitations of these rankings. Firstly, only the responses to BP and DMBA were included in the analyses. Secondly, dose-response data for both the carcinogen and/or promoter were not available for many of the mouse strains and stocks. Although these rankings represent subjective analyses, the differences between mice on the extremes of the rankings are significant.

Tumor Initiation

Whenever a known skin carcinogen has been appropriately tested, it has shown skin tumor-initiating activity (*8*). In a two-stage mouse skin system, initiation is the only stage that requires the presence of the carcinogen and the measured carcinogenic potency of a chemical reflects its capacity for tumor initiation. There is both a good qualitative and quantitative correlation between the complete carcinogenic and tumor initiating activities of several chemical carcinogens in mouse skin (*8*). This is true when one considers the number of papillomas per mouse at early times (10 to 20 weeks) or the final carcinoma incidence after tumor initiation (*8*).

It is possible that a carcinogen lacking promoting ability would not be detected when tested as a complete carcinogen. In this regard, however, we have found a number of chemical compounds such as benz(a)anthracene (BA), dibenz(a,c)anthracene, [DB(a,c)A], chrysene, urethan, BP-7,8-dihydrodiol-9,10-epoxide and BA-3,4-dihydrodiol-1,2-epoxide that have tumor-initiating activity but either lack or have very weak complete carcinogenic activity (*8*).

There is a good dose-response relationship of many carcinogens used as tumor initiators in the two-stage carcinogenesis system using SENCAR

Table 2. Comparison of complete carcinogenesis and initiation-promotion in various species.[a]

		Tumor histology			
Species	Treatment	Basal cell carcinomas	Carcinomas	No. of papillomas	Melanomas
Mouse	Complete		+ +	+	
	Two-stage		+	+ + + +	
Rat	Complete	+			
	Two-stage	+			
Hamster	Complete		+		+
	Two-stage				+

[a]Data of Slaga (*12*) and Phillips et. al. (*20*).

Table 3. Sensitivity to skin carcinogenesis in different stocks and strains of mice.[a]

Action	Order of sensitivity
Complete carcinogenesis	SENCAR>CD-1>C57BL/6>BALB/c>ICR/Ha Swiss>C3H
Two-stage carcinogenesis (initiation-promotion)	SENCAR>>CD-1>ICR/Ha Swiss>BALB/c>C57BL/6>C3H >DBA/2

[a]Data represent sensitivities to BP and DMBA. Rankings represent a subjective analysis because dose-response data were not available for many strains (*12,20*).

mice. This is illustrated in Table 4. A good dose-response relationship exists for DMBA and BP to initiate skin tumors in Sencar mice. As can be seen, a good correlation exists between the number of papillomas per mouse at 15 weeks and the final carcinomas incidenced at 50 weeks. The percent of mice with papillomas has also a reasonable correlation but the dose response is very narrow. The Sencar mouse was derived from crossing Charles River CD-1 mice with skin tumor sensitive mice (originally derived from Rockland mice) and selecting for sensitivity to DMBA-phorbol ester tumor promoter two-stage carcinogenesis for eight generations starting with the F_1 cross as described by Boutwell (*4*). The mice developing the earliest and most papillomas after initiation-promotion treatment were selected for each breeding. The Sencar mice are between 10 and 20 times more sensitive to DMBA tumor initiation than the CD-1 mice (*13*). However, the SENCAR mice are only between three and five times more sensitive to BP tumor initiation than the CD-1 mice (*13*). In addition, the Sencar mice are two to three times more sensitive to TPA promotion than the CD-1 (*13*).

There is even a greater difference in the sensitivity to two-stage skin carcinogenesis between Sencar and C57BL/6 mice. As pointed out above, the Sencar mouse is very sensitive to two-stage and complete carcinogenesis. C57BL/6 mice are very refractory to two-stage skin carcinogenesis by BP-TPA. As shown in Table 5, even high initiating doses of BP (1600 nmole) and high promoting doses of TPA (10 μg) are very ineffective in causing skin tumors (*12*). However, C57BL/6 mice do respond to complete carcinogenesis by BP (*10*). This unequal susceptibility to complete and two-stage carcinogenesis within a stock or strain of mice strongly suggests that the promotional phases of complete and two-stage carcinogenesis are dissimilar. In addition, differences in sensitivity to initiation and promotion between mice may be due to alterations in the promotional phase of two-stage carcinogenesis. In this regard, we have recently found that benzoyl peroxide is an effective promoter in C57BL/6 and Sencar mice (Slaga et al., unpublished data). The reason why TPA is not an effective promoter in C57BL/6 mice may be related to its lack of ability to induce a sustained hyperplasia (Davidson and Slaga, unpublished data).

The tumor initiation phase appears to be an irreversible step which probably involves a somatic cell

Table 4. Dose-response studies on the ability of DMBA and BP to initiate skin tumors in SENCAR mice.[a]

Initiator	Dose, nmole	No. of papillomas per mouse at 15 weeks	% of mice with papillomas at 15 weeks	% of mice with carcinomas at 50 weeks
DMBA	100	22.0	100	100
DMBA	10	6.8	100	40
DMBA	1	3.2	93	22
DMBA	0.1	0.5	20	5
BP	200	7.5	100	55
BP	100	3.2	78	30
BP	50	1.4	60	18

[a]The mice were treated 1 week after initiation with twice weekly applications of 5 μg of TPA (*8*).

Table 5. Initiation-promotion in SENCAR and C57BL/6 mice.

Treatment	Animal	Result	Dose response
TPA, repetitive, 52 weeks, no initiation	SENCAR mouse	5-20% papillomas <15% carcinomas	No
Benzoyl peroxide, repetitive, 52 weeks, no initiation	SENCAR mouse	<5% tumors	No
TPA, 52 weeks, after initiation	SENCAR mouse	Papillomas (early) Carcinomas (late)	Yes
Benzoyl peroxide after initiation	SENCAR mouse	Papillomas (early) Carcinomas (late)	Yes
TPA, repetitive, 52 weeks, no initiation	C57BL/6 mouse	None	–
Benzoyl peroxide, repetitive 52 weeks, no initiation	C57BL/6 mouse	None	–
TPA, repetitive, 52 weeks, initiation with 50-1600 nmole BP	C57BL/6 mouse	<5% papillomas <10% carcinomas	–
Benzoyl peroxide, repetitive, 52 weeks, after initiation	C57BL/6 mouse	45% carcinomas	

mutation as evidenced by a good correlation between the carcinogenicity of many chemical carcinogens and their mutagenic activities (*14-16*). Most tumor initiating agents either generate or are metabolically converted to electrophilic reactants, which bind covalently to cellular DNA and other macromolecules (*16*). Previous studies have demonstrated a good correlation between the carcinogenicity of several polycyclic aromatic hydrocarbon (PAH) and their ability to bind covalently (*8,17,18*). Table 6 summarizes our data which shows the strong correlation between the covalent binding of PAH to DNA and their tumor initiating activities.

As previously discussed, for any individual stock or strain of mouse, it has been generally observed that there is an excellent correlation between the amount of PAH bound to DNA and the skin tumor response. However, this correlation between DNA binding and tumor response breaks down when a comparison is made between mouse strains or stocks that differ in their tumor response to two-stage or to complete carcinogenesis (*19,20*). Phillips et al. (*19,20*) have demonstrated that the kinetics of binding of DMBA to the DNAs of C57BL/6, DBA/2 and Swiss mice were virtually identical. Although there is the possibility that a specific metabolite of the DMBA was responsible for the tumor response and was undetected in this study, recent investigations suggest that the major metabolites of DMBA and BP are qualitatively similar in mouse strains that vary in their response to two-stage or complete carcinogenesis with PAHs (*13*). Although these data are far from conclusive, they suggest that some aspects of initiation are probably similar in strains of mice that differ in their response to two-stage or complete carcinogenesis.

Inhibitors of Tumor Initiation

In order to help us better understand the mechanism of PAH carcinogenesis, we have been studying many compounds with the capacity to inhibit PAH tumor initiation. Potent inhibitors of skin tumor initiation in mice include: antioxidants [butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and selenium], flavones (7,8-benzoflavone, 5,6-benzoflavone and quercetin) vitamins A, C and E; certain noncarcinogenic polycyclic aromatic hydrocarbons [dibenz(a,c)anthracene, benz(a)anthracene, benzo(e)pyrene and pyrene]; environmental contaminants such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and polychlorobiphenyls (PCB); sulfur mustard; polyriboinosinic-polyribocytidylic acid (Poly I:C); and anti-inflammatory steroid.

Some of the flavones and antioxidants appear to inhibit skin carcinogenesis by inhibiting the metabolism of the carcinogen to its ultimate carcinogenic form (*21,22*). The antioxidants, butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), are widely used as food preservatives and have been shown to also inhibit lung, mammary, forestomach, colon and liver cancer in experimental animals induced by a wide range of chemicals (*23*). Similar inhibitory results have been noted for selenium and vitamins C and E (*8,21*). The noncarcinogenic PAHs and the environmental contaminants appear to inhibit skin carcinogenesis by inducing the metabolism of the carcinogen to detoxified products, thereby decreasing the binding of the PAH to DNA (*24,25*). This is epitomized by the environmental contaminants 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and polychlorobiphenyls (PCB) which are extremely potent inducers of PAH carcinogen metabolism and potent inhibitors of their carcinogenic effect (*26-28*). Although TCDD is one of the most toxic agents known, its inhibitory effect on PAH carcinogenesis is at nontoxic dose levels.

Sulfur mustard inhibits tumor initiation by actually killing the initiated cells (*29*). The polyinosinic:-polycytidylic acid (Poly I:C) and the anti-inflammatory steroids appear to inhibit tumor initiation by slowing down carcinogen metabolism by their antigrowth effect (*30,31*). Some of the above agents have been shown to inhibit carcinogenesis in a number of tissues and by a variety of chemical carcinogens, indicating they may be useful agents in the chemoprevention of cancer in man (*23*). In general, these inhibitors of skin tumor initiation act by (1) alteration of the metabolism of the carcinogen (decreased activation and/or increased detoxification), (2) scavenging of active molecular species of carcinogens to prevent their reaching the critical target site(s) in the cells or (3) competitive inhibition. In all cases this leads to a decrease in covalent binding to critical targets such as DNA. Table 7 reveals a good correlation in SENCAR or CD-1 mice between the ability of a number of compounds to inhibit tumorigenesis and their ability to inhibit the binding of the PAH to DNA.

Table 6. Correlation of ability of polycyclic aromatic hydrocarbons (PAHs) to bind covalently to epidermal DNA with the tumor initiating activity.[a]

PAHs	Relative ability to covalently bind to epidermal DNA	Relative tumor initiating activity
DMBA	10.0	10.0
MC	6.5	6.0
BP	3.3	2.0
DB(a,h)A	1.7	1.5
DB(a,c)A	0.8	0.2

[a]DMBA was given a value of 10 since it gave the maximum response in binding and to initiate tumors in a two-stage system of tumorigenesis. All the other PAHs are expressed as values relative to DMBA's response.

Tumor Promotion

Although the phorbol esters are the most potent of the mouse skin tumor promoters, a wide variety of other compounds have been shown to have skin tumor promoting activity, as shown in Table 8. After the phorbol esters and dihydroteleocidin B, anthralin is the most potent tumor promoter known of the compounds listed in Table 8. Van Duuren and co-workers have reported a fairly extensive structure-activity study with anthralin and derivatives (*32*). Likewise, Boutwell and co-workers (*33*) have reported a structure-activity study of a number of phenolic compounds which are weak promoters in comparison to the phorbol esters and anthralin. Although several of the other compounds shown in Table 8 have moderate to weak activity as tumor promoters there have not been any extensive structure-activity studies performed. We have recently found that benzo(e)pyrene (*25*) and benzoyl peroxide (*34*) are relatively good tumor promoters. In addition, Scribner and Scribner (*35*) reported that the moderate complete carcinogenic activity of 7-bromomethylbenz(a)anthracene was due to its strong promoting activity and weak initiating activity. Other free radical-generating compounds which are good skin tumor promoters include benzoyl peroxide, lauroyl peroxide, decanoyl peroxide, chloroperbenzoic acid, *p*-nitroperoxybenzoic acid and *tert*-butyl hydroperoxide. These agents were found not to have skin tumor initiating or complete carcinogenic activity (*34*).

The dose-response ability of TPA to promote tumors after DMBA initiation is shown in Table 9. As was the case for tumor initiation, there is also a very good dose-response relationship for tumor promotion when considering either the number of papillomas per mouse at 15 weeks or the percent of mice with squamous cell carcinomas at 50 weeks. Similar results have also been reported using SENCAR mice (*36*), Charles River CD-1 mice (*37*) or ICR/Ha Swiss mice (*38*).

In addition to causing inflammation and epidermal hyperplasia, the phorbol ester and other tumor promoters produce several other morphological and biochemical changes in skin as listed in Table 10. Of

Table 7. Correlation of various compounds to inhibit tumor initiation by DMBA with their abilities to inhibit covalent binding of DMBA to epidermal DNA.[a]

Inhibitors	Relative ability to inhibit DMBA tumor initiation by at least 50%	Relative ability to inhibit DMBA binding to by at least 50%
TCDD	100.0	100.0
DB(a,c)A	10.0	15.0
7,8-BF	5.0	8.0
B(e)P	5.0	3.0
BHA	0.2	0.1
BHT	0.1	0.1
Vitamin C	0.1	0.1

[a]TCDD was given a value of 100 since it gave the greatest inhibition of tumor initiation and DMBA binding to epidermal DNA. For example, TCDD at a 1 μg dose level almost completely inhibited DMBA tumorigenesis and DMBA binding to DNA. All the other compounds are expressed as values relative to TCDD's response. For example, BHA at a 1000 μg dose level inhibited DMBA tumor initiation and binding by at least 50%.

Table 8. Skin tumor promoters.[a]

Promoters	Potency
Croton oil	Strong
Certain phorbol esters found in croton oil	Strong
Some synthetic phorbol esters	Strong
Certain euphorbia latices	Strong
Anthralin	Moderate
Certain fatty acids and fatty acid methyl esters	Weak
Certain long chain alkanes	Weak
Phenolic compounds	Weak
Surface active agents (sodium lauryl sulfate, tween 60)	Weak
Citrus oils	Weak
Extracts of unburned tobacco	Moderate
Tobacco smoke condensate	Moderate
Iodoacetic acid	Weak
1-fluoro-2,4-dinitrobenzene	Moderate
Benzo(e)pyrene	Moderate
Benzoyl peroxide	Moderate
7-Bromoethylbenz(a)anthracene	Strong
Dihydroteleocidin B	Strong[b]

[a]Data of Slaga (*8*).
[b]Dihydroteleocidin B has promoting activity at doses similar to TPA (Slaga and Sugimura, unpublished data).

Table 9. Dose-response studies on the ability of TPA to promote tumors after DMBA initiation.[a]

Promoter	Dose, μg	Time to first papilloma, weeks	No. of papillomas per mouse at 15 weeks	% with papillomas at 15 weeks	% with carcinomas at 50 weeks
TPA	10	8	3.0	100	32
TPA	5	6	7.2	100	46
TPA	2	7	6.5	100	45
TPA	1	8	3.6	80	25
TPA	0.1	11	0.4	5	8

[a]The mice were initiated with 10 nmole of DMBA and promoted one week later with twice weekly applications of various dose levels of TPA (*12*).

Table 10. Morphological and biochemical responses of mouse skin to phorbol ester and other tumor promoters.[a]

Response
* Induction of inflammation and hyperplasia
Increase in DNA, RNA and protein synthesis
Initial increase in keratinization followed by a decrease
Increase in phospholipid synthesis
Increase in prostaglandin synthesis
Increase in histone synthesis and phosphorylation
* Increase in ornithine decarboxylase activity followed by increase in polyamines
Increase in histidine and DOPA decarboxylase activity[b]
Decrease in the isoproterenol stimulation of cAMP
Decrease in the number of dexamethasone receptors[b]
Decrease in SOD and catalase[b]
* Induction of embryonic state in adult skin
* 1. Induction of dark cells (primitive stem cells)
2. Induction of embryonic proteins in adult skin
3. Induction of morphological changes in adult skin resembling papillomas, carcinomas and embryonic skin
4. Decrease in histidase activity
5. Increase in protease activity
6. Decrease in response of G_1chalone in adult skin
7. Increase in cAMP independent protein kinase in adult skin resembling tumors and embryonic skin

[a]See Slaga et al. (*55*).
[b]Slaga et al., unpublished results.

the observed phorbol ester related effects on the skin, the induction of epidermal cell proliferation, ornithine decarboxylase (ODC) and dark basal keratinocytes have the best correlation with promoting activity (*39-44*). In addition to the induction of dark cells, which are normally present in large numbers in embryonic skin, there are many other embryonic conditions which appear in adult skin after treatment with tumor promoters (Table 10).

It is difficult to determine which of the many effects associated with phorbol ester tumor promotion are in fact essential components of the promotion process. A good correlation appears to exist between promotion and epidermal hyperplasia when induced by phorbol esters (*40*). However, other agents that induce epidermal cell proliferation do not necessarily promote carcinogenesis (*45*). However, it should be emphasized that all known skin tumor promoters do induce epidemal hyperplasia (*11*). O'Brien et al. (*39*) have reported an excellent correlation between the tumor promoting ability of various compounds (phorbol esters as well as nonphorbol ester compounds) and their ability to induce ODC activity in mouse skin. However, mezerein, a diterpene similar to TPA but with weak promoting activity, was found to induce ODC to levels that were comparable to those induced by TPA (*46*). Raick found that phorbol ester tumor promoters induced the appearance of "dark basal cells" in the epidermis, whereas ethylphenylpropiolate (EPP), a nonpromoting epidermal hyperplasic agent, did not (*41-43, 47*). Wounding induced a few dark cells which seemed to correlate with its ability to be a weak promoter (*41-43*). In addition, a large number of these dark cells are found in papillomas and carcinomas (*42,43*). Slaga et al (*44,48*) reported that TPA induced about three to five times the number of dark cells as mezerein which was the first major difference found between these compounds.

Inhibitors of Tumor Promotion

Various modifiers of the tumor promotion process have been very useful in our understanding of the mechanism(s) of tumor promotion. Table 11 lists the potent inhibitors of mouse skin tumor promotion by TPA. The anti-inflammatory steroid fluocinolone acetonide (FA) was an extremely potent inhibitor of phorbol ester tumor promotion in mouse skin (*49*).

Table 11. Inhibitors of phorbol ester skin tumor promotion.[a]

Inhibitor	Reference
Anti-inflammatory steroids	(*55*)
Cortisol	
Dexamethasone	
Fluocinolone acetonide	
Vitamin A derivatives	(*55*)
Combination of retinoids and anti-inflammatory agents	(*55*)
Protease inhibitors	(*55*)
Tosyl lysine chloromethyl ketone (TLCK)	
Tosyl arginine methyl ester (TAME)	
Tosyl phenylalanine chloromethyl ketone (TPCK)	
Antipain	
Leupeptin	
Cyclic nucleotides	(*55*)
Phosphodiesterase inhibitors, isobutylmethylxanthine (IBMX)	(*55*)
Dimethyl sulfoxide (DMSO)	(*55*)
Butyrate, acetic acid	(*55*)
Bacillus Calmette-Guerin (BCG)	(*55*)
Polyriboinosinic: Polyribocytidylic acid (Poly I:C)	(*55*)
Prostaglandin synthesis inhibitors	
5,8,11,14-Eicosatetraynoic acid (ETYA)	
Phenidone	
Thromboxane synthetase inhibitors	a
Imidazolacetophenone (RO22-3581)	
Imidazolphenol (RO22-3582)	
Phospholipase A_2 inhibitor	a
Dibromoacetophenone	
Arachidonic acid	a
Polyamine synthesis inhibitor	a
Difluoromethylornithine, DFMO	
Histamine	a
H_1 receptor inhibitor	a
Diphenhydramine	
Butylated hydroxyanisole (BHA)	a
Butylated hydroxytoluene (BHT)	a
Disulfiram	a
Hydroxyanisole	a

[a]Slaga et al, unpublished results.

Repeated applications of as little as 0.01 μg almost completely counteracted skin tumorigenesis. FA also effectively counteracts the induced cellular proliferation associated with application of phorbol ester tumor promoters. Certain retinoids are also potent inhibitors of mouse skin tumor promotion (*50*). Verma and co-workers (*50*) have shown that the retinoids that inhibit skin tumor promotion are potent inhibitors of phorbol ester-induced epidermal ODC activity. We have recently found that a combination of FA and retinoids produces an inhibitory effect on skin tumor promotion greater than that produced by each separately (*51*).

The work of Belman and Troll also indicates that protease inhibitors cyclic nucleotides, dimethyl sulfoxide and butyrate also inhibit mouse skin tumor promotion by phorbol esters (*52*). In addition to butyric acid, acetic acid also inhibits tumor promotion (*45,52*). The phosphodiesterase inhibitor isobutylmethylxanthine was also found to inhibit tumor promotion which gives further support to the inhibitory effect of cyclic nucleotides (Slaga and Weeks, unpublished results). Schinitsky and co-workers (*53*) reported the inhibitory effect of Bacillus Calmette-Guerin (BCG) vaccination on skin tumor promotion. It has been shown that Poly I:C has an inhibitory effect on carcinogenesis and tumor promotion (*30*). This appears to be mediated by its inhibition of promoter and carcinogen induced cell proliferation (*30*). Certain prostaglandin synthesis inhibitors, thromboxane synthesis inhibitors and phospholipase A_2 inhibitors also inhibit skin tumor promotion which suggest that prostaglandins and thromboxane may be important in tumor promotion (*54*). Although the mechanism is not presently understood, arachidonic acid at high doses is a potent inhibitor of tumor promotion (*54*). α-Difluoromethylornithine (DFMO), a specific inhibitor of polyamine synthesis also inhibits tumor promotion which suggests that polyamines are also important (*55*). The mechanism(s) by which histamine and diphenhydramine inhibit tumor promotion is currently not known (S. M. Fischer, unpublished results). Although BHA, BHT, disulfiram and *p*-hydroxyanisole are potent inhibitors of skin tumor promotion by both TPA and benzoyl peroxide, their mechanism of action is currently not known (T. J. Slaga, unpublished results). It is possible that free radicals are important in tumor promotion and thus these agents may prevent promotion by their free radical-scavenging ability.

Multistage Promotion

As previously discussed, mezerein, a diterpene similar to TPA, was capable of causing most of the morphological and biochemical changes in skin and in cells in culture that TPA does, but TPA was at least 50 times more active as a tumor promoter (*46*). A comparison of these TPA and mezerein responses are shown in Table 12. Clearly, mezerein is as potent or more potent than TPA. This is especially true regarding the induction of epidermal ODC and epidermal hyperplasia. The effect of mezerein on ODC activity suggests that ODC induction is not a critical event in tumor promotion (*46*). It should be emphasized that this conclusion is also true for the other morphological and biochemical responses to mezerein.

Table 12. Comparison of cellular and biochemical responses to TPA and mezrein[a]

	Relative response	
	TPA	Mezerein
Enhancement of neoplastic phenotype	100	100
Promotion of neoplastic transformation (C3H-10T-1/2)	100	80
Induction of epidermal cellular proliferation	50	100
Comitogenesis in lymphocytes	100	100
Inhibition of differentiation in friend erythroleukemia cells	100	100
Stimulation of DNA synthesis	50	100
Stimulation of ODC activity	80	100
Stimulation of plasminogen activator production	20	100
Stimulation of epidermal histidine decarboxylase	20	100
Induction of dark basal keratinocytes	100	25
Tumor promotion	100	2
Relative binding to receptor	100	2

[a]For a comparative purpose the maximum response of mezerein or TPA is expressed as a 100. The values should only be considered as an approximation.

Because of the many similarities in morphological and biochemical responses induced by TPA and mezerein, we felt that mezerein, although a weak promoter, would be a good candidate as a compound to be used in the second stage of a two-stage promotion protocol as originally reported by Boutwell (*4*). We recently reported that mezerein was a potent stage II promoter (*10*). A summary of the results on the use of mezerein as a second stage promoter in two-stage promotion are shown in Table 13. As illustrated, TPA is about 50 times more active as a promoter than mezerein. When 2 μg of TPA is given twice weekly for only 2 weeks after DMBA initiation, no tumors are induced, compared to twice weekly treatments for 18 weeks. However, when mezerein is given at a dose of either 1, 2, 4 or 6 μg twice weekly after the limited TPA treatment, it induced a significant tumor response in a dose-dependent manner. The ability of mezerein to act as a potent second stage promoter was repeated in more than 15 separate experiments (*10,55,56*). Also shown in Table 13 is the ineffectiveness of EPP as a com-

plete promoter and as a second stage promoter. In addition, we recently found that 4-O-methyl TPA, the calcium ionophore A23187, hydrogen peroxide and wounding which do not promote are effective first-stage promoters (Tables 13 and 14). These compounds or wounding induce epidermal hyperplasia and increase the number of dark basal keratinocytes (*57*). Table 14 shows some of the characteristics of the first and second stages of promotion. Besides showing a good dose-response for TPA as a first stage promoter only a single application of TPA is necessary for stage I of promotion to be expressed after repeated applications of mezerein. In addition stage I of the promotion is partially irreversible for four weeks. As previously stated stage II of promotion requires multiple applications and also shows a good dose response with mezerein or 12-deoxyphorbol-13-2,4,6-decatrienoate (DPtri-D).

The effectiveness of some of the inhibitors of tumor promotion on two-stage promotion was recently reported by this laboratory (*56*). The effects of FA, retinoic acid (RA), DFMO and tosyl phenylalanine chloromethylketone (TPCK) on two-stage promotion are shown in Table 15. FA was a potent inhibitor of stage I and II of promotion but to a greater degree for stage I than stage II. It should be emphasized that only four applications of FA with TPA were necessary to counteract the tumor response. RA was ineffective in stage I but was a potent inhibitor of stage II promotion whereas TPCK specifically inhibited stage I but not stage II. These experiments were repeated several times and were very reproducible (*55,56*). Recently,

Table 14. Characteristics of the first and second stages of tumor promotion.

Stage	Characteristic
I	Good dose response exists for TPA Only one application of TPA is necessary Partially irreversible Four weeks can separate first and second stages of promotion without a decrease in tumor response There is an 80% decrease in tumor response if 10 weeks separate stage I and stage II of promotion Nonpromoting agents [calcium ionophase (A23187), 4-0-methyl TPA, H_2O_2 and wounding] can act as stage I promoters
II	Good dose-response exists for mezerein Multiple applications are required Nonpromoting agents (DPtri-D) can act as stage II promoters

Table 13. Two-stage promotion.[a]

		Initiation	Promotion: Stage I			Promotion: Stage II			Relative tumor response
1	DMBA	1 wk	TPA		32 times				100
2	DMBA	1 wk	Mezerein	(4 μg)	32 times				2
3	DMBA	1 wk	TPA		4 times	Acetone		28 times	0
4	DMBA	1 wk	TPA		4 times	Mezerein	(1 μg)	28 times	35
5	DMBA	1 wk	TPA		4 times	Mezerein	(2 μg)	28 times	50
6	DMBA	1 wk	TPA		4 times	Mezerein	(4 μg)	28 times	85
7	DMBA	1 wk	TPA		4 times	Mezerein	(6 μg)	28 times	120
8	DMBA	1 wk	4-0-methyl TPA	(80 μg)	4 times	Mezerein	(2 μg)	28 times	40
9	DMBA	1 wk	TPA		4 times	4-0-methyl TPA	(80 μg)	28 times	0
10	DMBA	1 wk	A23187	(80 μg)	4 times	Mezerein	(2 μg)	28 times	60
11	DMBA	1 wk	TPA		4 times	A23187	(80 μg)	28 times	0
12	DMBA	1 wk	EPP	(14 mg)	32 times				1
13	DMBA	1 wk	TPA		4 times	EPP	(14 mg)	28 times	2

[a]The mice were initiated with 10nmole of DMBA and promoted with 2 μg of TPA or as shown above (*55*).

Table 15. Effects of tumor promotion inhibitors on two-stage promotion.[a]

		Initiation	Promotion: Stage I		Promotion: Stage II		Tumor response (% of control)
1	DMBA	1 wk	TPA	4 times	Mezerein	28 times	100
2	DMBA	1 wk	TPA + FA	4 times	Mezerein	28 times	0
3	DMBA	1 wk	TPA	4 times	Mezerein + FA	28 times	20
4	DMBA	1 wk	TPA + RA	4 times	Mezerein	28 times	95
5	DMBA	1 wk	TPA	4 times	Mezerein + RA	28 times	20
6	DMBA	1 wk	TPA + TPCK	4 times	Mezerein	28 times	25
7	DMBA	1 wk	TPA	4 times	Mezerein + TPCK	28 times	94

[a]The mice were initiated with 10nmole of DMBA and promoted with 2 μg of TPA and 2 μg of mezerein. FA (1 μg), RA (10 μg) and TPCK (10 μg) were applied simultaneously with TPA or mezerein (*55*).

Weeks and Slaga (unpublished results) found that DFMO was a potent specific inhibitor of stage II promotion.

Since the only major morphological or biochemical difference between the effects of TPA and mezerein on the skin is the ability of TPA to induce a large number of dark basal keratinocytes (*44,56*), we were interested in determining the effects of various inhibitors of promotion on the appearance of these dark cells. We reasoned that if these dark cells are critical in the first stage of promotion and if FA and TPCK are potent inhibitors of stage I and RA and DFMO of stage II, then FA and TPCK should counteract the appearance of these cells, whereas RA and DFMO should not. The results of FA, RA, DFMO and TPCK on the induction of dark basal keratinocytes by TPA are summarized in Table 16. As hypothesized, FA and TPCK were found effectively to counteract the appearance of the dark cells induced by TPA, whereas RA and DFMO had no effect (*44*).

Since TPCK inhibited stage I of promotion but not stage II, and since TPCK counteracted the TPA-induced increase in the dark basal keratinocytes but did not have any effect on TPA-induced hyperplasia, we were interested in determining the effect of TPCK on TPA-induced ODC activity. As shown in Table 16, TPCK had very little effect on TPA- and mezerein-induced epidermal ODC activity.

The anti-inflammatory steroid FA not only counteracted the appearance of dark cells induced by TPA but also suppressed the hyperplasia induced by TPA. In fact, the skins of mice treated with FA plus TPA appeared the same as untreated skin. This is in agreement with our previously reported observations on the inhibitory effect of FA on TPA induced inflammation, hyperplasia and DNA synthesis (*49*). However, FA had little effect on the TPA increased ODC activity (Table 16) as compared to its effect on inhibition of promotion.

It is also of interest to point out that although RA inhibited stage 2 of promotion, it had no inhibitory effect on the TPA- or mezerein-induced hyperplasia (Table 16). However, certain retinoids have been found to be potent inhibitors of TPA- and mezerein-induced epidermal ODC activity (*46*). In this regard, DFMO is a specific irreversible inhibitor of ODC activity. This data suggests that the induction of epidermal ODC activity followed by increased polyamines may be important in stage II of promotion. In this regard FA and TPCK have either no effect or only a slight inhibitory effect on TPA or mezerein induced ODC activity (*55*). FA does, however, significantly decrease the TPA induced spermidine levels in the epidermis (*55,56*). This effect plus FA's inhibitory effect on TPA-induced hyperplasia may be responsible for its inhibitory effect on stage II promotion. Figure 2 depicts the various stages of promotion, the important events in each stage, and where the various inhibitors are effective.

In conclusion, skin carcinogenesis can be operationally and mechanistically divided into at least three stages; initiation, stage I of promotion and

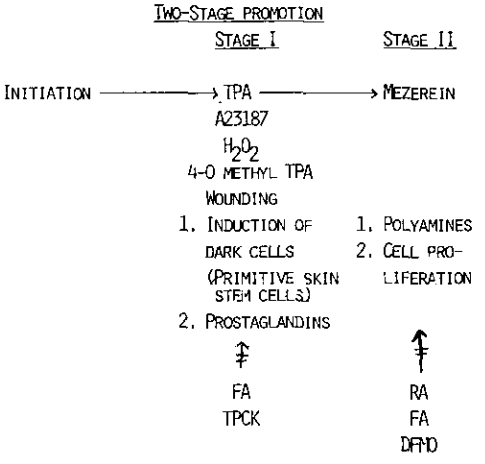

FIGURE 2. Diagram of the various stages of skin carcinogenesis showing the important events in stage I and II of promotion and where FA, RA, DFMO and TPCK inhibit promotion.

Table 16. Effects of FA, RA and TPCK on tumor promotion and TPA-induced epidermal hyperplasia, dark keratinocytes and polyamine levels.

	Relative ability (%) to counteract[a]			
Inhibitor	TPA promotion	TPA-induced hyperplasia	TPA-induced dark cells	TPA-induced ODC and polyamine levels
FA	*100*	*100*	*100*	*20*
RA	80	0	0	85
TPCK	70	0	70	10
DFMO[b]	55	0	0	95

[a]The abilities of FA, RA, DFMO and TPCK to counteract the various TPA responses are expressed from 100% (complete suppression) to 0% (no effect). The effects of the inhibitors were determined from dose-response studies (*55, 56*).

[b]Weeks and Slaga, unpublished data.

stage 2 of promotion. Covalent binding of the initiator to epidermal DNA probably in dark basal keratinocytes leading to a mutation in some aspect of differentiation appears to be important in the initiation stage. The stimulation of dark basal keratinocytes (stem cells?) are important in stage I of promotion whereas polyamines and cell proliferation are important in stage 2 of promotion. It is not presently known if other experimental carcinogenesis systems or the induction of human cancer go through a series similar to that in the mouse skin.

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