Staurosporine Induces Protein Kinase C Agonist Effects and Maturation of Normal and Neoplastic Mouse Keratinocytes in Vitro

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ABSTRACT

Staurosporine is a potent but nonselective inhibitor of protein kinase C (PKC) and blocks responses to 12-O-tetradecanoylphorbol-13-acetate (TPA) in several cell types in vitro. In cultured primary mouse keratinocytes, however, staurosporine fails to inhibit TPA-mediated keratinocyte maturation and itself elicits responses that are similar to TPA (T. Sako et al., Cancer Res., 48: 4646–4650, 1988). After exposure to 10 nM staurosporine for 24 h, essentially all keratinocytes undergo morphological differentiation, whereas 160 nM TPA induces this response in about 50% of epidermal cells. These concentrations of staurosporine and TPA cause a 4–5-fold induction of epidermal transglutaminase activity and cornified envelopes, both markers of the terminal stage of keratinocyte differentiation. Staurosporine, but not TPA, also induces morphological and biochemical maturation in 2 neoplastic mouse keratinocyte cell lines, 308 and SP-1. The ability of staurosporine to elicit the same responses as TPA suggested that it may be functioning paradoxically as a PKC agonist in intact keratinocytes. In support of this hypothesis, staurosporine induces ornithine decarboxylase activity, inhibits 125I-labeled epidermal growth factor binding, and induces expression of c-fos mRNA. Downregulation of PKC by pretreatment of primary keratinocytes with 60 nM bryostatin partially blocks staurosporine-mediated induction of cornified envelopes and inhibition of 125I-labeled epidermal growth factor binding, implicating PKC in these responses. The ability of staurosporine to mimic and/or enhance certain responses to TPA suggests that this agent is acting as a functional PKC agonist in cultured keratinocytes.

INTRODUCTION

The mouse skin carcinogenesis model has yielded insights into both early events in the development of epithelial neoplasia and later changes associated with neoplastic progression (1). The ability to cultivate keratinocytes in vitro has further facilitated analysis of both genetic and epigenetic events that are important in cutaneous neoplasia (2, 3). For example, the tumor promoter TPA1 is a potent inducer of maturation in cultured primary mouse keratinocytes but not neoplastic cells (4–6). This differential response in vitro may provide insight into the mechanism by which phorbol esters promote tumor formation in vivo. Repeated application of TPA to initiated mouse skin is associated with accelerated maturation and desquamation of normal keratinocytes, which permits the clonal expansion of differentiation-resistant initiated cells into a papilloma (7). An important prediction of this hypothesis is that restoring the ability of neoplastic keratinocytes to differentiate would render them nontumorigenic. PKC consists of a family of phospholipid-dependent kinases that phosphorylate proteins at serine and threonine residues (8). Since phorbol esters bind and activate PKC (9), this enzyme family has been implicated in TPA-mediated responses such as the induction of differentiation markers in cultured primary keratinocytes. Several classes of inhibitors have been used in an attempt to better understand the functions of PKC; unfortunately, all of these also inhibit other protein kinases, which complicates the interpretation of results using intact cells. One of the most potent inhibitors presently available is staurosporine (10), which inhibits PKC at nanomolar doses in vitro by interacting with its catalytic domain (11, 12). Whereas staurosporine blocks the effects of TPA in several nonepithelial cell types (13–16), it fails to inhibit TPA-mediated maturation in primary mouse keratinocytes and induces certain responses characteristic of TPA exposure (13).

In the present study, we have extended the analysis of staurosporine in primary mouse keratinocytes as well as 2 neoplastic mouse keratinocyte cell lines, 308 and SP-1 (17). Our findings indicate that staurosporine, unexpectedly, functions primarily as a PKC agonist in cultured mouse keratinocytes.

MATERIALS AND METHODS

Materials. Staurosporine was purchased from Calbiochem, La Jolla, CA; TPA from LC Services, Woburn, MA; [3H]putrescine, [14C]ornithine, and [125I]labeled EGF from New England Nuclear, Boston, MA; and EGF (receptor grade) from Collaborative Research, Bedford, MA. Bryostatin (bryostatin 1) was generously provided by Dr. G. R. Pettit, Arizona State University, Tempe, AZ.

Cell Culture. Primary epidermal keratinocytes were isolated from newborn BALB/c mice as described (18). The neoplastic keratinocyte cell line 308 was established from BALB/c mouse skin initiated with 7,12-dimethylbenz[a]anthracene in vivo, the SP-1 cell line from papillomas produced on Sencar mice by initiation with 7,12-dimethylbenz[a]anthracene and TPA promotion (17). Both 308 and SP-1 cells form benign papillomas when grafted onto the backs of immune-deficient mice (17). Primary as well as neoplastic keratinocytes were cultured in Eagle’s minimum essential medium containing 8% chexeled fetal calf serum and 1% antibiotic/antimycotic solution (18). Unless otherwise indicated, the concentration of Ca2+ in the medium was adjusted to 0.05 mM to maintain a basal cell-like population of undifferentiated cells (19).

Enzyme Assays. Activity of epidermal transglutaminase was determined by measuring cross-linking of [3H]putrescine to dimethylsacine (20). Cell lysates were incubated at 37°C for 10 min to inactivate tissue transglutaminase. Ornithine decarboxylase activity of cell lysates was determined by quantifying release of 14CO2 from [14C]ornithine (21).

Cornified Envelope Quantitation. Keratinocytes were analyzed for cornified envelope formation as described (22), with minor modifications. Floating cells were harvested by collecting culture medium from each 60-mm dish and combining with 2 washes using PBS. Cells were pelleted for 5 min at 1000 rpm in a benchtop clinical centrifuge and resuspended in 100 μl of lysis buffer (2% SDS and 20 mM dithiotreitol in PBS). Attached cells were harvested in 250 μl lysis buffer, combined with the preparation of floating cells, and incubated at 95°C for 10 min. The nonsolubilized, intact cornified envelopes, characteristic of terminally differentiated keratinocytes, were counted using a hemocytometer (22).
125I-labeled EGF Binding. EGF binding was assessed in 6-well tissue culture dishes plated at a cell density of 2.5 x 10^6 cells/well (23). After treatment, cultures were washed twice with binding buffer (Dulbecco's minimum essential medium with 50 mM N,N-bis-(2-hydroxyethyl)-2-amino sulfuric acid (pH 6.8) and 1 mg/ml bovine serum albumin) at 4°C, then incubated with 1 ml binding buffer containing 125I-labeled EGF (1.4 x 10^6 dpm) for 4-6 h on a bed of ice (23). A second set of cultures received, in addition, 1 µg/ml unlabeled EGF to assess nonspecific binding of radioactive ligand. Following the incubation period, cultures were washed 4 times with ice-cold binding buffer, harvested in 1.5 ml lysis buffer (0.1 M Tris (pH 7.4), 0.5% SDS, 1 mM EDTA), and radioactivity determined by scintillation counting.

RNA Isolation and Northern Blot Analysis. RNA was isolated by ultracentrifugation of guanidine isothiocyanate lysates through a 5.7 M cesium chloride gradient (24); 20 µg of total RNA were loaded per lane and electrophoresed through a 1% agarose gel containing 0.66 M formaldehyde (25). RNA was blotted to reinforced nitrocellulose membrane (BA-S NC; Schleicher & Schuell, Keene, NH) and baked at 80°C in a vacuum oven for 2 h. Filters were prehybridized at 42°C overnight in buffer containing 50% formamide as described previously (19); approximately 20 x 10^6 dpm of probe were added and hybridization was carried out overnight. The final wash was performed at 65°C using 0.2 x standard saline-citrate with 0.1% SDS. Filters were exposed to Kodak X-Omat AR film at -70°C with intensifying screens. Mouse c-fos transcript was detected using a 1.2 kilobase Bgl II to Sal I fragment from the Finkel-Biskis-Jinkins murine sarcoma virus sequence (26). Following autoradiography, remaining probe was removed by washing in 1% glycerol in deionized water at 80°C for 2 min (25) and the filter was rehybridized to a full-length rat glyceraldehyde phosphate dehydrogenase cDNA in a pUC18 vector (27). Probes were labeled by random priming to a specific activity of 2 x 10^6 cpm/µg DNA.

Miscellaneous Assays. Cell toxicity was determined using trypan blue exclusion as a marker for viable cells. Cells growing in 60-mm culture dishes were washed once with PBS, incubated at room temperature with 1 ml 0.4% trypan blue in normal saline (GIBCO, Rockville, MD), washed twice with PBS, then placed on ice until cells were counted. Protein in cell lysates was determined using a commercial colorimetric assay (Bio-Rad Laboratories, Richmond, CA).

RESULTS

Both TPA and Staurosporine Induce Maturation of Primary Keratinocytes in Vitro. Primary keratinocytes grown in 0.05 mM Ca^2+ medium exhibit a basal cell-like phenotype with well-defined intercellular spaces and a characteristic cobblestone-like appearance (Fig. 1). The addition of 10 nM staurosporine or 160 nM TPA results in a rapid alteration of cell morphology that is first detectable after 20 min. While both agents induce an elongated morphology by 3 h, cells exposed to TPA are more extensively altered and frequently exhibit attenuated, dendrite-like processes (Fig. 1). After 24 h, about 50% of the cells in TPA-treated cultures have rounded up and detached from the substrate (Fig. 1): this is a characteristic response in keratinocytes that are pharmacologically induced to differentiate in medium with 0.05 mM Ca^2+ and does not reflect nonspecific cytotoxicity (4). In contrast to TPA, nearly all cells in cultures exposed to staurosporine for 24 h have detached from the substrate leaving behind a few small, bipolar cells (Fig. 1). Many remaining cells in TPA-treated cultures are immunoreactive for keratin 14, whereas none is positive in staurosporine-treated cultures (data not shown), indicating that staurosporine induces detachment of essentially all keratinocytes from the substrate. To determine if the morphological response to 10 nM staurosporine is caused by direct cytotoxicity, primary keratinocytes exposed to staurosporine were monitored by trypan blue exclusion. In DMSO controls and cultures treated with ≤10 nM staurosporine, <5% of the cells took up trypan blue. Not unexpectedly, substantial cell damage could be obtained at higher staurosporine doses, however, with 74% of cells trypan blue “positive” after a 7.5-h exposure to 1 µM staurosporine (data not shown). These findings indicate that the morphological response to 10 nM staurosporine occurs in the absence of gross cytotoxicity.

Epidermal transglutaminase activity is elevated in terminally differentiating keratinocytes both in vivo and in vitro (20, 28).
The addition of either staurosporine or TPA to 8-day-old primary keratinocytes results in a dose-dependent induction of epidermal transglutaminase (Fig. 2). Staurosporine induces a 4-fold increase in enzyme activity at 10 nM; TPA a 5-fold increase at 100 and 1000 nM (Fig. 2). The induction of transglutaminase activity by staurosporine is tightly restricted to a dose of 10 nM, while TPA-mediated activation occurs at doses ranging from 10 nM to 10 μM. The absence of transglutaminase induction at higher staurosporine doses is consistent with a toxic effect at concentrations of 100 nM or more while the 10 nM dose elicits a programmed response.

Activation of epidermal transglutaminase results in the assembly of rigid, detergent-insoluble cornified envelopes characteristic of terminally differentiated keratinocytes (29, 30). To further evaluate the ability of staurosporine to induce keratinocyte maturation, cornified envelope formation was assessed in 7-day-old primary keratinocyte cultures exposed to medium with 0.1% DMSO, 10 nM staurosporine, or 160 nM TPA for 24 h. Staurosporine induces a 5-fold increase in cornified envelopes relative to control cultures; TPA a 4-fold increase (Fig. 3). The induction of both epidermal transglutaminase activity and cornified envelope formation by TPA and staurosporine indicates that both agents induce maturation in cultured primary mouse keratinocytes.

Staurosporine but not TPA Induces Maturation of Neoplastic Keratinocytes in Vitro. A basic defect of neoplastic keratinocytes is their inability to differentiate in response to either TPA or elevated extracellular Ca²⁺, providing them with a potential growth advantage over normal cells in vivo (31). Since staurosporine induces differentiation in nearly the entire population of primary mouse keratinocytes exposed to this agent, we were interested in determining whether similar responses could be induced in neoplastic cells. The 2 cell lines selected for analysis, designated 308 and SP-1, cannot be induced to terminally differentiate in vitro and produce benign papillomas when grafted onto the backs of nude mice (17). After a 24-h exposure to staurosporine, both 308 and SP-1 cells exhibited morphological changes similar to those seen in primary cells; in contrast, exposure to TPA did not appreciably alter the appearance of either cell line when compared with DMSO-treated controls (Fig. 4). TPA was ineffective at inducing morphological differentiation at doses ranging from 1.6 nM to 16 μM (data not shown). Although the overall response pattern to staurosporine is the same in 308 and SP-1 cells as it is in primary keratinocytes, the kinetics of this process are different: treatment for at least 48 h is required for a maximal response in the cell lines compared with 24 h for primary keratinocytes.

Epidermal transglutaminase was assayed to determine if staurosporine induces this differentiation marker in neoplastic keratinocytes as it does in primary cells. Exposure of SP-1 cells to staurosporine caused a dose-dependent increase in enzyme activity, with a maximum 50-fold induction at 100 nM (Fig. 5). As with primary keratinocytes, higher doses were ineffective. In contrast to staurosporine, TPA had no detectable effect on epidermal transglutaminase activity at any dose ranging from 100 pM to 10 μM (Fig. 5).

Cornified envelope formation was also determined in cultures of staurosporine-treated neoplastic cells. Both neoplastic cell lines produced cornified envelopes when exposed to staurosporine, but not TPA. There was a 10-fold induction of cornified envelopes by staurosporine in SP-1 cells and a 60-fold induction in 308 cells (Fig. 6). Consistent with the results for epidermal transglutaminase activity, cornified envelope formation was not induced in TPA-treated cultures. These data indicate that staurosporine is a potent inducer of maturation in neoplastic keratinocyte cell lines that are entirely defective in their response to either TPA or Ca²⁺ as inducers of differentiation.

Staurosporine Induces Certain Other Responses Characteristic of PKC Agonists. The ability of TPA and other phorbol esters to induce epidermal transglutaminase and cornified envelope formation in primary keratinocytes suggests that PKC activation is involved in this process. The induction of similar responses by staurosporine, a PKC inhibitor, was therefore unexpected. We have taken 2 approaches to further explore the potential involvement of PKC in staurosporine-mediated responses in keratinocytes: (a) cells rendered deficient in PKC were analyzed for their ability to respond to staurosporine; results from similar studies assessing effects of TPA have strongly implicated PKC in various responses to phorbol esters; and (b) the ability of staurosporine to elicit several additional responses associated with PKC activation was examined: induction of ornithine decarboxylase, inhibition of 125I-labeled EGF binding, and expression of c-fos mRNA.

Staurosporine-mediated Cornification Is Partially Blocked in PKC-deficient Primary Keratinocytes. Bryostatin is an ultrapotent PKC modulator that interacts with the phorbol ester bind-
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Fig. 4. Staurosporine (STSP) induces morphological differentiation in neoplastic keratinocyte cell lines. 308 and SP-1 keratinocyte cell lines were exposed to 0.1% DMSO, 160 nM TPA, or 10 nM STSP for 24 h. Note lack of response to TPA. Similar results were obtained in 3 additional experiments with each cell line. × 65.

Both Staurosporine and TPA Inhibit $^{125}$I-labeled EGF Binding in Primary Keratinocytes. Exposure of a variety of cultured cell types to TPA results in inhibition of EGF binding, which is attributed to PKC-mediated phosphorylation of the EGF receptor (40). TPA and staurosporine rapidly inhibit binding of $^{125}$I-labeled EGF to primary keratinocytes: exposure to 160 nM TPA causes a 94% reduction, and 10 nM staurosporine a 72% reduction relative to controls (Fig. 9). As noted above for other parameters, the lack of response to 1 μM staurosporine may reflect cytotoxicity at this high dose.

To assess the role of PKC in the inhibition of $^{125}$I-labeled EGF binding in primary keratinocytes, PKC-deficient cells were generated by exposing cultures to 60 nM bryostatin for 1 day. Both TPA- and staurosporine-mediated inhibition of $^{125}$I-labeled EGF binding was inhibited over a 4-h period: bryostatin pretreatment completely blocked the response to TPA, whereas it blocked the response to staurosporine by an average of 50% (Fig. 10). These findings suggest that PKC is involved in the inhibition of $^{125}$I-labeled EGF binding in response to both TPA and staurosporine.

Both TPA and Staurosporine Induce c-fos mRNA. TPA rapidly induces transcription of the protooncogene c-fos, which is a useful marker for PKC activation in a variety of cells (41). In primary mouse keratinocytes, TPA causes a sustained elevation of steady-state c-fos mRNA during an 8-h treatment (Fig. 11). In cultures exposed to staurosporine, c-fos is also induced but with different kinetics and to a lesser extent than TPA. The greatest induction is at 8 h of staurosporine treatment (Fig. 11), although elevated levels are detected at both 1 and 6 h after longer exposures (data not shown). Combined exposure to both staurosporine and TPA induces higher expression of c-fos mRNA than TPA alone (Fig. 11). These results again indicate both the inability of staurosporine to block certain TPA-mediated responses and the mimicry of TPA that it can induce.
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Fig. 6. Staurosporine (STSP) induces cornified envelopes in neoplastic keratinocytes. 308 and SP-1 cells were exposed to 0.1% DMSO, 160 nM TPA, or 10 nM STSP in 1.4 mM Ca\(^{2+}\) medium. After 3 days, cells were harvested and cornified envelopes isolated.

Fig. 7. Down-regulation of PKC partially inhibits cornified envelope formation in primary keratinocytes exposed to staurosporine (STSP). Seven-day-old primary keratinocytes were cultured with or without 60 nM bryostatin (+BRYO) for 15 h. Fresh medium with 10 nM STSP or 160 nM TPA, ± BRYO, was added for an additional 46 h and cornified envelopes isolated. Columns, average value from duplicate dishes; bars, range. In an additional experiment, BRYO pretreatment reduced cornified envelope formation by 70% in both STSP and TPA-treated cultures.

DISCUSSION

We have shown that the protein kinase inhibitor staurosporine elicits several of the same responses in cultured mouse keratinocytes as TPA, a PKC activator. In primary keratinocytes, both agents elicit similar changes in cell morphology, elevate epidermal transglutaminase activity, and induce formation of cornified envelopes, responses that are associated with keratinocyte differentiation. More importantly, staurosporine induces similar changes in the neoplastic cell lines 308 and SP-1, both of which fail to differentiate in response to either TPA or elevations in extracellular Ca\(^{2+}\). Although the mechanism by which staurosporine induces keratinocyte maturation is uncertain, we present evidence indicating that it may be acting, in part, as a functional PKC activator in intact keratinocytes.

In PKC-deficient (bryostatin pretreated) keratinocytes, induction of cornified envelope formation is blocked in both TPA-treated and staurosporine-treated cultures (Fig. 7), indicating that an intact PKC pathway is necessary for these responses to both agents. Induction by staurosporine of several other responses attributed to PKC activation provides additional sup-
GAPDH mRNA were identified using 32P-labeled DNA probes as described in either inhibited (43) or enhanced (42, 43) by staurosporine. There are conflicting reports regarding effects on ODC activity in published data (42). The failure to demonstrate this response in keratinocytes, staurosporine at relatively high doses enhances 125I-labeled EGF binding in A431 cells and Swiss 3T3 fibroblasts (46), illustrating the different responses to this agent in different target cells. The ability of staurosporine to induce low-level expression of c-fos in primary keratinocytes is also consistent with its proposed role as a PKC agonist (Fig. 11). Enhancement of c-fos transcript levels in cultures exposed to both staurosporine and TPA, compared with those exposed to TPA alone, argue against its functioning as a PKC inhibitor for this response (Fig. 11). In contrast to our findings, staurosporine was noted to inhibit TPA-mediated c-fos expression in JB6 cells (47) and HeLa cells (48), although these data were not shown. Staurosporine's ability to induce TPA-responsive genes is not limited to c-fos. In LLC-PK1 cells, both TPA and staurosporine induce urokinase mRNA in a dose-dependent manner, with an additive effect when combined (49). Both agents also induce expression of mRNA encoding neurotensin and neuromedin N in PC12 cells (50).

While it is clear that staurosporine elicits many TPA-like responses in cultured primary keratinocytes, this may not be the case for all responses. In preliminary experiments using digitonin-permeabilized keratinocytes (51), TPA enhanced the phosphorylation of a band migrating at ~M, 40,000, consistent with reports from other laboratories (52, 53). In contrast, staurosporine failed to enhance phosphorylation of this band and appeared to block this response to TPA when the agents were combined. This result is consistent with another study in which staurosporine blocked TPA-mediated phosphorylation of a M, 34,000 substrate in cultured keratinocytes (45). Unlike the results discussed thus far, which indicate PKC agonist activity, this observation suggests that staurosporine may also have PKC inhibitory activity.

The mechanism by which staurosporine induces multiple responses associated with PKC activation is not known. Since cultured mouse keratinocytes express mRNA coding for PKC α, δ, ε, γ, and η (54), and each isozyme may elicit a unique set of biological responses, a potential mechanism accounting for our findings is that staurosporine is selectively influencing one or more PKC isozymes. This may be occurring either by a direct effect of staurosporine on PKC, resulting in enzyme activation, or indirectly by modulation of a regulatory factor involved in PKC-mediated signal transduction. Regarding the first possibility, staurosporine has been reported to inhibit association of PKC with membranes both in vitro (55) and in intact cells (49). Thus, translocation of an isozyme not inhibited by staurosporine to a permissive microenvironment in intact keratinocytes could result in its net activation. A selective effect of staurosporine on different PKC isozymes is suggested by the report that K252a, a structurally related compound, has an IC50 for PKC α, β, and γ (56) that is 2 orders of magnitude higher than for mouse brain PKC (57). Alternatively, staurosporine could elicit PKC agonist responses indirectly by affecting the metabolism of diacylglycerols, phospholipids, or intracellular Ca2+ levels. For example, diacylglycerol kinase may be an important regulator of PKC activity by converting diacylglycerol, the endogenous PKC activator, into

the inactive metabolite phosphatidic acid (58). Preferential inhibition of diacylglycerol kinase activity by staurosporine could result in elevated levels of diacylglycerol that could trigger PKC activation. Staurosporine may also cause activation of PKC by inhibiting a kinase that normally maintains the enzyme in an inactive state. Another potential explanation for our findings is based on the report that TPA-mediated dephosphorylation of c-Jun enhances its ability to bind DNA (48). Staurosporine’s inhibition of a kinase that normally maintains c-Jun in its fully phosphorylated, inactive state could result in enhanced DNA-binding and AP-1 activity, mimicking this response to TPA.

Although we have assumed that keratinocyte maturation in response to either TPA or staurosporine is related to PKC activation, an alternative interpretation should be considered. Since TPA rapidly down-regulates PKC in cultured mouse keratinocytes, it is possible that cornification is associated with depletion of PKC rather than its activation. If this is the case, staurosporine could be mimicking TPA by directly inhibiting PKC. This possibility seems unlikely since bryostatin, an agent that also down-regulates PKC in cultured keratinocytes, does not induce cornification and blocks staurosporine effects. In addition, we have been unable to induce cornification in primary keratinocytes using 2 other PKC inhibitors, H7 and calphostin (data not shown).

The ability of staurosporine to mimic TPA-mediated maturation in cultures of primary keratinocytes suggests that it may have tumor-promoting activity. In fact, staurosporine has been reported to inhibit promotion by TPA or teleocidin (44, 59) and itself possess promoting activity (44). These conflicting findings, as well as those reported for staurosporine-mediated changes in ODC activity both in vivo and in vitro (see above), may be dose-related. The strict dose-dependence we have demonstrated for staurosporine-mediated responses in vitro suggests the presence of multiple targets that are affected at different drug concentrations. If this is the case, relatively small differences in the amount of drug delivered to target cells could markedly alter the biological responses elicited both in normal cells and in potential tumor cells in vivo. Staurosporine’s ability to inhibit several classes of protein kinases with IC50 ranging from 3 to 61 nM (60) is consistent with this hypothesis.

Despite the similar responses to staurosporine and TPA in cultures of primary keratinocytes, only staurosporine induces maturation of neoplastic cells that are unresponsive in this regard to either TPA or Ca2+. Studies are currently under way to determine whether altered expression or function of PKC isozymes could account for the differences in responsiveness of neoplastic cells to TPA and staurosporine. The unique ability of staurosporine to induce maturation of neoplastic keratinocytes suggests that it may also have antitumor properties in vivo. In preliminary studies using 308 and SP-1 cells in a skin grafting model on nude mice, topical application of staurosporine to induce maturation of neoplastic cells to TPA and staurosporine. The unique ability of staurosporine to induce maturation of neoplastic keratinocytes in vitro suggests that it may provide a useful new approach for the treatment of epithelial neoplasms.

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