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Two Novel Glycosides from the Fruits of *Morinda Citrifolia* (Noni) Inhibit AP-1 Transactivation and Cell Transformation in the Mouse Epidermal JB6 Cell Line[1]

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ABSTRACT

The fruit juice of *Morinda citrifolia* (noni), a plant originally grown in the Hawaiian and Tahitian islands, has long been used by islanders to treat diseases, including cancer. Two novel glycosides, 6-*O*-(β-D-glucopyranosyl)-1-*O*-octanoyl-β-D-glucopyranose and asperulosidic acid, extracted from the juice of noni fruits, were used to examine their effects on 12-*O*-tedtradecanoylphorbol-13-acetate (TPA)- and epidermal growth factor (EGF)-induced AP-1 transactivation and cell transformation in mouse epidermal JB6 cells. The results indicated that both compounds were effective in suppressing TPA- or EGF-induced cell transformation and associated AP-1 activity. TPA- or EGF-induced phosphorylation of c-Jun, but not extracellular signal-regulated kinases or p38 kinases, was also blocked by the compounds, indicating that c-Jun N-terminal kinases were critical in mediating TPA- or EGF-induced AP-1 activity and subsequent cell transformation in JB6 cells.

INTRODUCTION

Morinda citrifolia (Rubiaceae), known as "noni" locally, is a plant grown in the Hawaiian and Tahitian islands. The stem, bark, root, leaf, and fruits of the plant have been used traditionally by islanders as medicines to treat a broad range of diseases, including diabetes, hypertension, and cancer (1, 2). The fruit juice of *Morinda citrifolia* or noni contains a polysaccharide-rich substance called noni-ppt that has been reported to have antitumor activity in the Lewis lung peritoneal carcinoma model (3). Noni-ppt administration significantly prolonged the survival duration of inbred Lewis lung tumor-bearing mice (1). Noni-ppt was suggested to suppress tumor growth through its regulation of the host immune system (4). It was reported to stimulate the release of several potential mediators, including TNF-α,[3] IL-1β, IL-10, IL-12 p70, IFN-γ, and nitric oxide (4). Some individual compounds from noni juice were reported to function as *ras* inhibitors and thus suppressed *ras*-expressing tumors (2). Improved survival time and curative effects occurred when noni-ppt was combined with suboptimal doses of standard chemotherapeutic agents, which suggests important clinical applications of noni-ppt as a supplemental agent in cancer treatment (4).

To elucidate the mechanism of the antitumorigenic effects of noni-ppt, we studied the effects of compounds isolated from noni-ppt on AP-1 activity and cellular transformation in mouse epidermal JB6 cells. AP-1 is an inducible eukaryotic transcription factor containing products of the *jun* and *fos* oncogene families (5–7). The inducible AP-1 complexes are composed of Jun-Jun or Jun-Fos dimers. When stimulated, AP-1 binds to transactivation promoter region TREs (TPA response elements) and induces transcription of several genes involved in cell proliferation, metastasis, and metabolism (8). Many stimuli are able to induce AP-1 activity (5), including the phorbol ester TPA, and EGF. These are the two most commonly used experimental stimuli used to activate AP-1 and induce cellular transformation in many different cell types and animal models (9). Increased AP-1 activity is associated with malignant transformation and cancer promoting agents, such as UV radiation (10), growth factors (11), phorbol esters (12, 13), and transforming oncogenes (11). In JB6 mouse epidermal cell lines, TPA and EGF were shown to induce AP-1 transcriptional activity in promotion-sensitive (P^+), but not in promotion-resistant (P^-), cellular phenotypes (12). When AP-1 induction was blocked, P^+ cells reverted to the P^- phenotype, indicating that AP-1 activity is required for TPA- or EGF-induced cell transformation (12). On the other hand, inhibition of AP-1 activity has been shown to lead to suppression of cell transformation (14). Some chemopreventive agents, including aspirin, sodium salicylate, tea polyphenols, perillyl alcohol, and retinoic acid, have been reported to inhibit cell transformation and tumor promotion and were also found to suppress AP-1 transactivation (14–20). All of these results strongly indicate that the inhibition of AP-1 activity leads to the suppression of tumor promotion.

Reports focusing on chemopreventive effects of the fruits of noni are limited. Here we used two novel compounds, NB10 (Fig. 1*A*) and NB11 (Fig. 1*B*), isolated from the juice of noni fruits to examine their effects on AP-1 transactivation and subsequent cell transformation in mouse epidermal JB6 cells. Because AP-1 has an important role in tumorigenesis, the results of this investigation may provide new insights into the mechanism of noni juice in tumor suppression and the possibility for its application in tumor prevention and treatment.

MATERIALS AND METHODS

Cell Culture and Reagents. AP-1 luciferase reporter plasmid stably transfected mouse epidermal JB6 P^+ 1–1 and the JB6 P^+ mouse epidermal cell line, Cl 41, were cultured in monolayers at 37°C, 5% CO_2 using Eagle's MEM containing 5% FBS, 2 mM L-glutamine, and 25 μg of gentamicin/ml. FBS and MEM were from Bio Whittaker, Inc. (Walkersville, MD); PD169316, TPA, aprotinin, and leupeptin were from Sigma Chemical Co. (St. Louis, MO); EGF was from Clonetics (San Diego, CA); NB10 and NB11 were extracted, and the structures were determined as described previously (2, 21); the luciferase assay substrate was from Promega (Madison, WI); and antibodies against ERKs or p38 kinase, specific antibodies against phosphorylated sites of ERKs, p38 kinase, and the JNK assay kit were from New England Biolabs, Inc. (Beverly, MA).

Luciferase Assay for AP-1 Transactivation. Confluent monolayers of JB6 P^+ 1–1 cells were trypsinized, and 8000 viable cells suspended in 100 μl of 5% FBS MEM were added to each well of a 96-well plate. Plates were incubated at 37°C in a humidified atmosphere of 5% CO_2. When cells reached 80–90% confluence, they were starved by culturing them in 0.1% FBS MEM

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[3] The abbreviations used are: TNF, tumor necrosis factor; NB10, 6-*O*-(β-D-glucopyranosyl)-1-*O*-octanoyl-β-D-glucopyranose; NB11, asperulosidic acid; AP-1, transcription activator protein 1; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; EGF, epidermal growth factor; ERK, extracellular signal regulated kinase; JNK, c-Jun N-terminal kinase; IL, interleukin; FBS, fetal bovine serum; EMSA, electrophoretic mobility shift assays; PMSF, phenylmethylsulfonyl fluoride; dThd, thymidine; MAPK, mitogen-activated protein kinase.

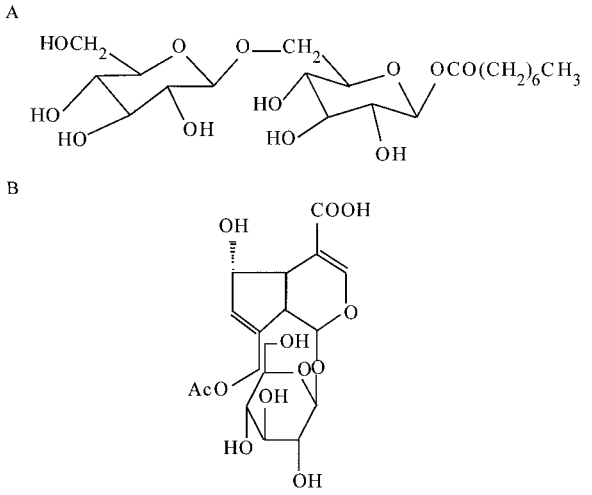

Fig. 1. *A*, NB10; *B*, NB11.

for another 24 h. The cells were then treated for 30 min with different concentrations of NB10 or NB11 as indicated and then exposed to TPA (20 ng/ml) or EGF (20 ng/ml) for 24 h. After treatment, cells were extracted with 100 μl of lysis buffer [0.1 M potassium phosphate buffer (pH 7.8); 1% Triton X-100; 1 mM DTT; 2 mM EDTA], and luciferase activity was measured using a luminometer (Monolight 2010). The results are expressed as relative AP-1 activity (22).

Anchorage-independent Transformation Assay. The effect of NB10 or NB11 on TPA- or EGF-induced cell transformation was investigated in JB6 C1 41 cells. Cells (8×10^3/ml) were exposed to TPA or EGF with or without NB10, NB11 (10–150 μM), or PD169316 (0.05–0.2 μM) in 1 ml of 0.33% Basale Medium Eagle agar containing 10% FBS >3.5 ml of 0.5% Basale Medium Eagle agar medium containing 10% FBS. The cultures were maintained in a 37°C, 5% CO_2 incubator for 4 weeks, and the cell colonies were scored as described by Colburn *et al.* (23). The effects of the compounds on cell transformation of JB6 C1 41 cells are presented as a percentage inhibition of cell transformation compared with TPA- or EGF-stimulated cells in soft agar.

AP-1 DNA Binding Studies. EMSA was performed essentially as described (24). Nuclear protein extracts were prepared from JB6 C1 41 cells by the modified method of Monick *et al.* (25). Briefly, JB6 C1 41 cells were cultured in 10-cm dishes and starved in 0.1% FBS MEM at 37°C in a 5% CO_2 incubator as described earlier. After a 24 h starvation, the cells were treated for 30 min with different concentrations of NB10, NB11, or PD169316 as indicated and then exposed to TPA (20 ng/ml) or EGF (20 ng/ml) for another 12 h. Then the cells were harvested and disrupted in 500 μl of lysis buffer A [25 mM HEPES (pH 7.8), 50 mM KCl, 0.5% NP-40, 100 μM DTT, 10 μg/ml leupeptin, 25 μg/ml aprotinin, and 1 mM PMSF]. After a 1-min centrifugation ($16,000 \times g$, 4°C), the pellet containing the nuclei was washed once with 500 μl of buffer B (buffer A without NP-40). The pellet containing the nuclei was resuspended in 150 μl of extraction buffer (buffer B but with 500 mM KCl and 10% glycerol) and shaken at 4°C for 30 min. The nuclear extracts were stored at −70°C until analysis. The DNA binding reaction (for EMSA) was carried out at room temperature for 30 min in a mixture containing 4 μg of nuclear protein, 1 μg of poly (dI-dC), and 15,000 cpm of ^{32}P-labeled double-stranded AP-1 oligonucleotide (5′-CGCTTGATGAGTCAGCCGGAA-3′). The samples were fractionated through a 5% polyacrylamide gel. Gels were dried and analyzed using the Storm 840 Phospho-Image System (Molecular Dynamics).

JNK Assay. JB6 Cl 41 cells were cultured and starved in 0.1% FBS MEM at 37°C in a 5% CO_2 incubator as described earlier. The cells were treated for 30 min with different concentrations of NB10 or NB11 and then exposed to TPA (20 ng/ml) or EGF (20 ng/ml) followed by culturing for another 30 min. The JNK assay was carried out according to the protocol of New England Biolabs, Inc. In brief, the cells were washed once with ice-cold PBS and disrupted in 300 μl of lysis buffer [20 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM Na PP_i, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 mg/ml leupeptin, and 1 mM PMSF] per sample. The lysates were sonicated and centrifuged, and 250 μl of the supernatant fraction were incubated with 2 μg of the N-terminal c-Jun (1–89) fusion protein bound to glutathione-Sepharose beads. The mixed samples were gently rocked overnight at 4°C. The beads were then washed twice with 500 μl of lysis buffer with PMSF and twice with 500 μl of the kinase buffer [25 mM Tris (pH 7.5), 5 mM β-glycerophosphate, 2 mM DTT, 0.1 mM Na_3VO_4, and 10 mM $MgCl_2$]. The kinase reactions were carried out at 30°C for 30 min in the presence of 100 μM ATP. c-Jun phosphorylation was selectively measured by Western immunoblotting using a chemiluminescent detection system (enhanced chemiluminescence) and a specific c-Jun antibody against phosphorylation of c-Jun at Ser63.

Immunoblotting for Phosphorylated JNKs, ERKs, and p38 Kinase. Immunoblotting for the phosphorylated proteins of JNKs, ERKs, and p38 kinase was carried out using specific MAPK antibodies against phosphorylated sites of JNKs, ERKs, or p38 kinase (14), according to the manufacturer's recommendations. Antibody-bound proteins were detected by chemiluminescence (enhanced chemiluminescence; New England Biolabs, Inc.) and analyzed using the Storm 840 Phosphor-Image System (Molecular Dynamics). JNKs, ERKs, and p38 kinase were detected by specific MAPK antibodies against JNKs, ERKs, and p38 as inner controls.

Statistical Analysis. Significant differences in AP-1 activity were determined using the Student *t* test. The results are expressed as means ± SD.

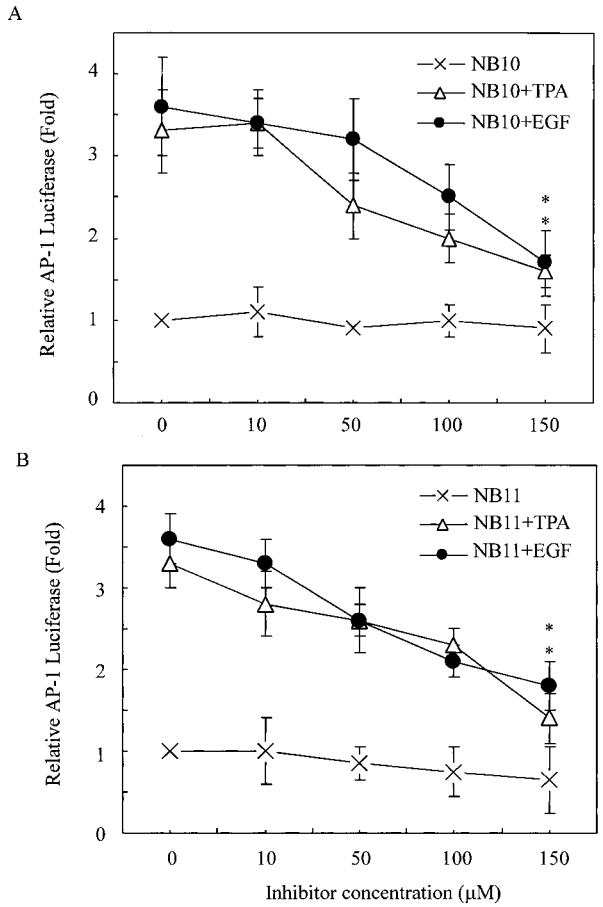

Fig. 2. NB10 or NB11 suppress both TPA- and EGF-induced AP-1 activity. Stably transfected JB6 P^+1–1 AP-1 luciferase reporter cells were cultured as described in "Materials and Methods." The cells were starved in 0.5% FBS/MEM and treated with different concentrations of NB10 or NB11 as indicated. After a 30-min treatment of the cells with NB10 or NB11, 20 ng/ml of TPA or EGF were or were not added, and the cells were cultured for another 24 h before harvest. AP-1 activity is expressed as a fold increase of relative luciferase units as assessed by luminometer. In *A*, TPA or EGF treatment induced a 3.3- or 3.6-fold increase in AP-1 activity, respectively, and NB10 (150 μM) significantly inhibited AP-1 activity [47%; *, a significant ($P < 0.01$) inhibition compared with TPA or EGF treatment with no inhibitor present; *bars*, SD of triplicate experiments, four wells each]. NB10 alone had no effect ($P > 0.05$; *bars*, SD of triplicate experiments, four wells each) on basal AP-1 activity. In *B*, NB11 (150 μM) significantly inhibited TPA- or EGF-induced AP-1 activity [53 or 49%, respectively; *, a significant ($P < 0.01$) inhibition compared with EGF treatment with no inhibitor present; *bars*, SD of triplicate experiments, four wells each]. NB11 alone caused a slight but insignificant ($P > 0.05$; *bars*, SD of triplicate experiments, four wells each) reduction of basal AP-1 activity.

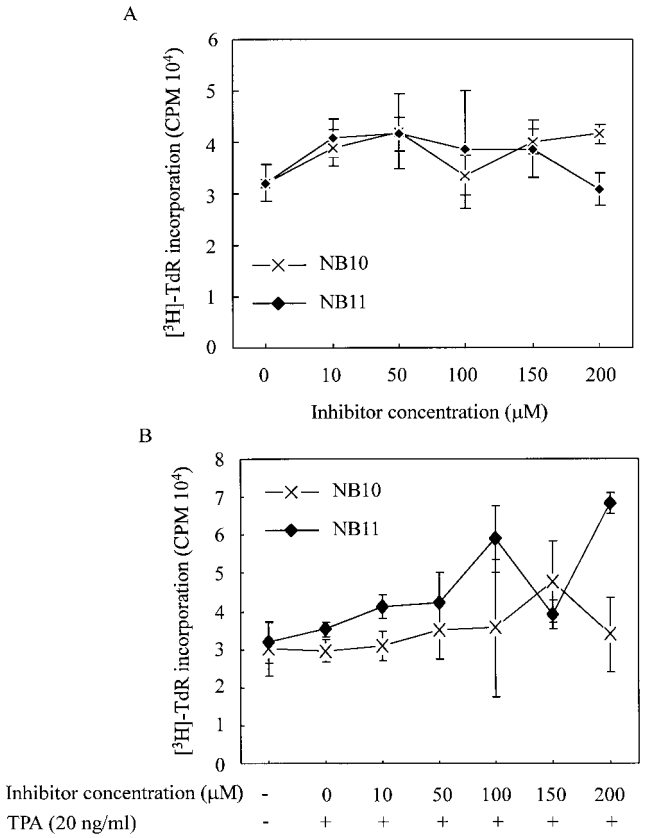

Fig. 3. The effects of NB10 or NB11 with or without TPA on JB6 cell proliferation. JB6 P^+1–1 cells (5×10^3) were seeded in 96-well plates. After culturing overnight, the cells were treated with the concentration of NB10 or NB11 indicated for 1 h, and then 20 ng/ml of TPA were or were not added. After culturing another 24 h in a 37°C, 5% CO_2 incubator, [^{3}H]dThd (0.5 μCi/well, 1 μCi = 37 GBq) was added to each well. The cells were harvested 12 h later, and incorporation of [^{3}H]dThd was measured by liquid scintillation counting. The results are presented as cpm. In *A*, no significant difference in [^{3}H]dThd incorporation could be detected at any concentration of NB10 or NB11 compared with control medium ($P > 0.05$; *bars*, SD of six wells). In *B*, NB10 or NB11 also did not cause a reduction of [^{3}H]dThd incorporation in TPA-treated cells.

RESULTS

NB10 and NB11 Suppress AP-1 Activity in JB6 Cells. The AP-1 complex not only mediates cell transformation and tumorigenesis, but it also appears to be involved in a broad range of physiological functions, including cell proliferation, survival, and apoptosis (26). To examine if NB10 or NB11 influences AP-1 activity and to exclude the possibility that either compound alone may affect the induction of AP-1, we investigated the effect of NB10 or NB11 on AP-1 activity in AP-1 luciferase reporter plasmid stably transfected mouse epidermal JB6 P^+ 1–1 cells. We observed a slight but insignificant ($P > 0.05$) inhibition of basal AP-1 activity by NB11 compared with untreated cells (Fig. 2). When JB6 cells were pretreated 30 min with NB10 or NB11 at the concentration indicated, both TPA- (Fig. 3*A*) and EGF-induced AP-1 activity were significantly inhibited by 150 μM NB10 (Fig. 2*A*) or NB11 (Fig. 2*B*; $P < 0.01$). DMSO used to dissolve the compounds had no effect on stimulated AP-1 activity (data not shown), and NB10 or NB11 caused no significant growth inhibition in the range of concentrations used in these experiments (Fig. 3*A*). Moreover, neither NB10 nor NB11 caused a reduction of [^{3}H]dThd incorporation in TPA-treated cells (Fig. 3*B*).

NB10 and NB11 Suppress Anchorage-independent Cell Transformation. Both TPA- and EGF-induced cell transformation on soft agar were significantly suppressed by NB10 or NB11 (Fig. 4, *A* and *B*). NB10 appeared to be more effective at the concentrations of 10, 50, and 100 μM (Fig. 4*A*, $P < 0.01$, indicated by $*$) than NB11 (Fig. 4*B*) in suppressing TPA-induced cell transformations on soft agar. NB10 and NB11 inhibited EGF-induced cell transformations only at the highest concentration (150 μM, Fig. 4*B*). The inhibitory effects of both compounds on AP-1 activity agree with their inhibitory effects on cell transformation. Therefore, these results suggest that the inhibition of cell transformation by the compounds is through their suppression of AP-1 activity.

NB10 and NB11 Repress AP-1 DNA Binding. In its function as a transcription factor, AP-1 mediates gene expression induced by TPA, growth factors, cytokines, and other stimuli (27). AP-1 DNA binding activity was assessed by EMSA (27), and results indicated that TPA (20 ng/ml; Fig. 5, *A* and *B*) or EGF (20 ng/ml; Fig. 5*D*) induced a significant increase in AP-1 DNA binding. The DNA binding was specific for AP-1 because a 10-fold excess of unlabeled AP-1 probe successfully competed with the labeled probe (Fig. 5*A*, *Lane 1*). NB10 or NB11 (100 μM) alone had little effect on AP-1 binding (Fig. 5*A*, *Lanes 3* and *4*, respectively). Both NB10 and NB11 successfully blocked TPA- or EGF-induced AP-1 DNA binding activity (Fig. 5, *B* and *D*, *Lanes 3–6*). A compound concentration of 100 μM was much more effective than 50 μM in blocking TPA- or EGF-induced AP-1 DNA-binding activity. Although these results somewhat reflected the inhibitory effects of NB10 and NB11 on AP-1 transactivation and cell transformation, they were not in total agreement. This was not surprising because AP-1 transactivation and DNA binding activity do not always mirror one another in their ability to

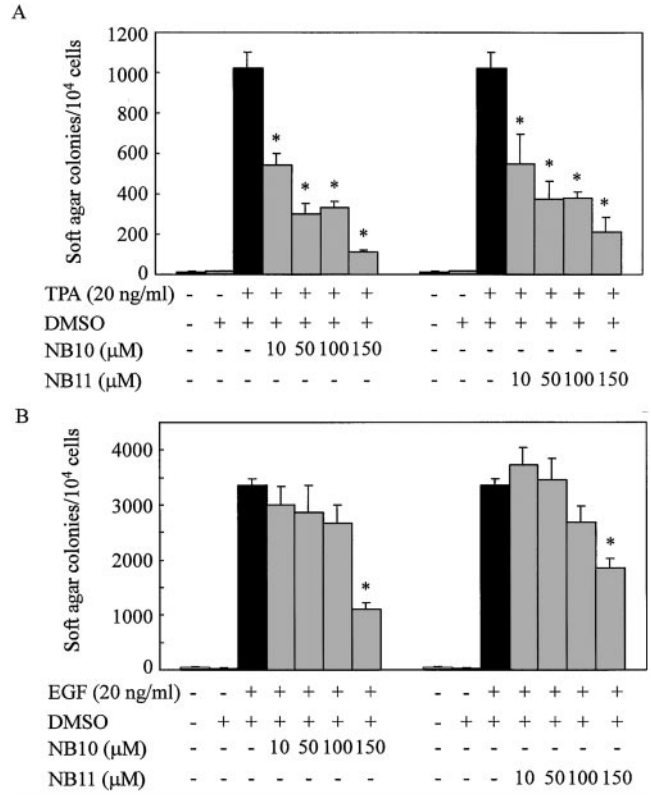

Fig. 4. NB10 or NB11 suppresses both TPA- and EGF-stimulated cell transformation on soft agar. JB6 Cl 41 cells seeded in triplicate wells were exposed to 20 ng/ml of TPA or EGF with or without NB10 or NB11 at the concentrations indicated and as described in "Materials and Methods." Cell colonies were scored after 4 weeks of incubation in a 37°C, 5% CO_2 atmosphere. In *A*, NB10 or NB11 effectively inhibited TPA-induced cell transformation on soft agar in a dose-dependent manner [$*$, a significant ($P < 0.01$) inhibition compared with TPA treatment with no inhibitor present; *bars*, SD of three wells each concentration]. In *B*, both compounds inhibited EGF-induced cell transformation but only at the highest concentration tested [150 μM; $*$, a significant ($P < 0.01$) inhibition compared with EGF treatment with no inhibitor present; *bars*, SD of three wells each concentration].

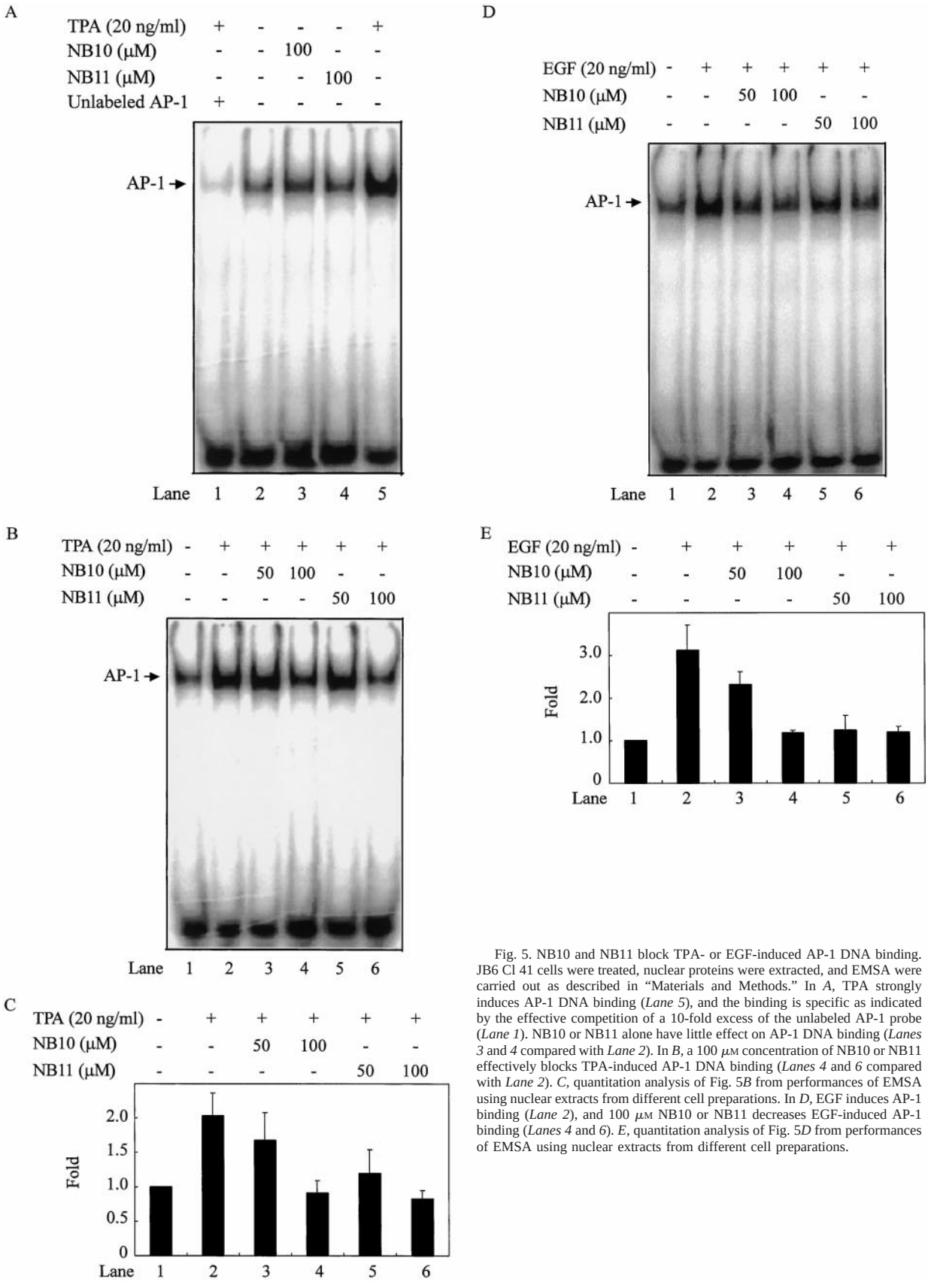

Fig. 5. NB10 and NB11 block TPA- or EGF-induced AP-1 DNA binding. JB6 Cl 41 cells were treated, nuclear proteins were extracted, and EMSA were carried out as described in "Materials and Methods." In *A*, TPA strongly induces AP-1 DNA binding (*Lane 5*), and the binding is specific as indicated by the effective competition of a 10-fold excess of the unlabeled AP-1 probe (*Lane 1*). NB10 or NB11 alone have little effect on AP-1 DNA binding (*Lanes 3* and *4* compared with *Lane 2*). In *B*, a 100 μM concentration of NB10 or NB11 effectively blocks TPA-induced AP-1 DNA binding (*Lanes 4* and *6* compared with *Lane 2*). *C*, quantitation analysis of Fig. 5*B* from performances of EMSA using nuclear extracts from different cell preparations. In *D*, EGF induces AP-1 binding (*Lane 2*), and 100 μM NB10 or NB11 decreases EGF-induced AP-1 binding (*Lanes 4* and *6*). *E*, quantitation analysis of Fig. 5*D* from performances of EMSA using nuclear extracts from different cell preparations.

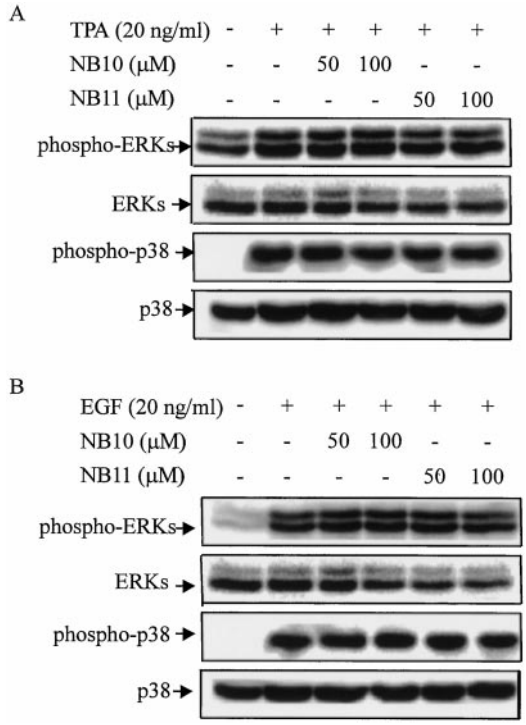

Fig. 6. Neither NB10 nor NB11 have an effect on TPA- or EGF-induced phosphorylation of ERKs or p38 kinase. JB6 Cl 41 cells were treated, and Western blot analysis was carried out as described in "Materials and Methods." Treatment with either TPA (*A*) or EGF (*B*) induces phosphorylation of ERKs or p38 kinase, and treatment with NB10 or NB11 had no effect on that phosphorylation (*A* and *B*).

activate transcription of target genes (27). Still, the reduced AP-1 DNA binding activity induced by higher concentrations of NB10 or NB11 may have a role in the mechanism leading to inhibition of AP-1 transactivation.

TPA- or EGF-induced Phosphorylation of ERKs and p38 Kinases Was Not Inhibited by NB10 or NB11. ERKs have been suggested to be most efficiently stimulated by growth factors and phorbol esters, whereas p38 kinase was shown to be induced by growth factors but not phorbol esters (27). In the current experiment, both TPA and EGF induced a strong phosphorylation of ERKs and p38 kinases (Fig. 6, *A* and *B*). However, NB10 or NB11 had no effect on the phosphorylation of either ERKs or p38 kinases (Fig. 6, *A* and *B*), indicating that ERKs and p38 kinases are not the targets of these compounds that lead to suppression of AP-1 activity in JB6 cells.

NB10 and NB11 Suppress TPA- or EGF-induced Phosphorylation of c-Jun. MAPKs contribute to the induction of AP-1 activity in response to a broad range of extracellular stimuli (27). Among the three types of MAPKs, JNKs are suggested to mediate the effects of UV irradiation or TNF (27). In the present experiment, we did not observe phosphorylation of JNKs induced by TPA or EGF (data not shown). However, both TPA and EGF induced an increase in JNKs activity that was measured by the phosphorylation of c-Jun, a direct substrate of JNKs (Fig. 7, *A* and *B*). NB10 or NB11 (50–100 μM) diminished TPA- or EGF-stimulated phosphorylation of c-Jun (Fig. 7, *A* and *B*). Because this method is more sensitive and more accurate in reflecting JNK activity *in vivo* (28, 29), the results suggested that the inhibition of c-Jun phosphorylation is one of the mechanisms contributing to the suppression of AP-1 activity and subsequent cell transformation by NB10 and NB11.

PD169316 Suppresses AP-1 DNA Binding and Anchorage-independent Cell Transformation. PD169316 is a specific inhibitor of MAPK p38. However, the compound is also known to inhibit phosphorylation of JNKs (Fig. 8; Refs. 30 and 31). To further determine whether JNK is important in mediating AP-1 activity and cell transformation, we used PD169316 to block AP-1 DNA binding and cell transformation induced by TPA or EGF in JB6 cells. The compound effectively blocked TPA- or EGF-induced AP-1 DNA binding and anchorage-independent cell transformation at the same concentrations in which it blocked JNK phosphorylation (Figs. 9 and 10).

DISCUSSION

Morinda Citrifolia (noni) is one of a variety of plants that have been used locally in the Hawaiian and Tahitian islands to treat a variety of diseases, including cancer, for hundreds or thousands of years (1–4). Two glycosides, one novel and one known, NB10 and NB11 respectively, were recently isolated from the fruits of noni (2, 21). NB10 was obtained as a white powder (21), and its structure is shown in Fig. 1*A*. NB11 (Fig. 1*B*) was isolated as a colorless oil (2), which has been found earlier in many other plants (32). Pharmacological studies

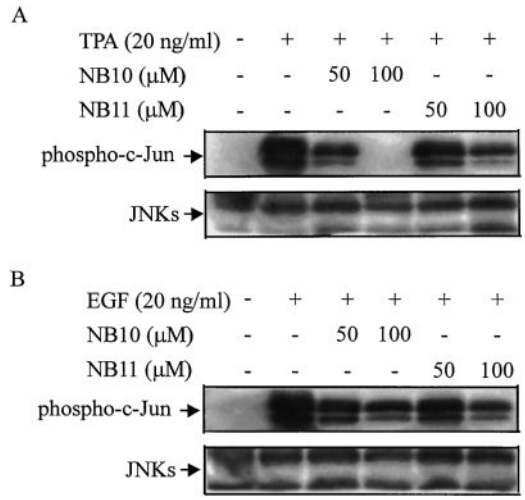

Fig. 7. NB10 and NB11 block TPA- or EGF-induced JNK activity. JB6 Cl 41 cells were treated with NB10 or NB11 and exposed to TPA or EGF as described in "Materials and Methods." JNK activity was determined by using the N-terminal c-Jun (1–89) fusion protein bound to glutathione-Sepharose beads to selectively "pull down" the JNK protein from cell lysates. The kinase reaction was carried out in the presence of 100 μM ATP, and phosphorylation of c-Jun, a direct substrate of JNK, was determined by Western immunoblotting using a specific antibody against phosphorylation of c-Jun at Ser63. Both TPA (*A*) and EGF (*B*) induce phosphorylation of c-Jun at Ser63, and both NB10 and NB11 markedly block the TPA- or EGF-induced phosphorylation. JNK by Western immunoblotting was applied as controls for the same volume of proteins in cell lysates when pulled down together with c-Jun (1–89) fusion protein bound to glutathione-Sepharose beads.

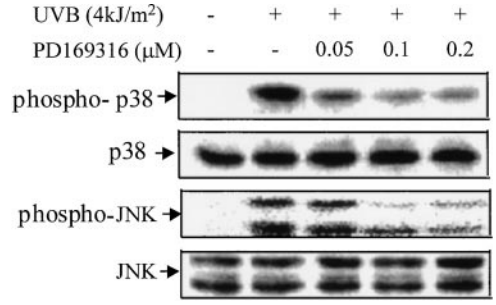

Fig. 8. PD169316 blocks UVB-induced phosphorylation of p38 or JNK kinase. JB6 Cl 41 cells were treated, and Western blot analysis was carried out as described in "Materials and Methods." Treatment with UVB (4 kJ/m^2) induces phosphorylation of p38 or JNK, and 0.05 μM or 0.1 μM PD169316 effectively blocks UVB-induced phosphorylation of p38 or JNK, respectively.

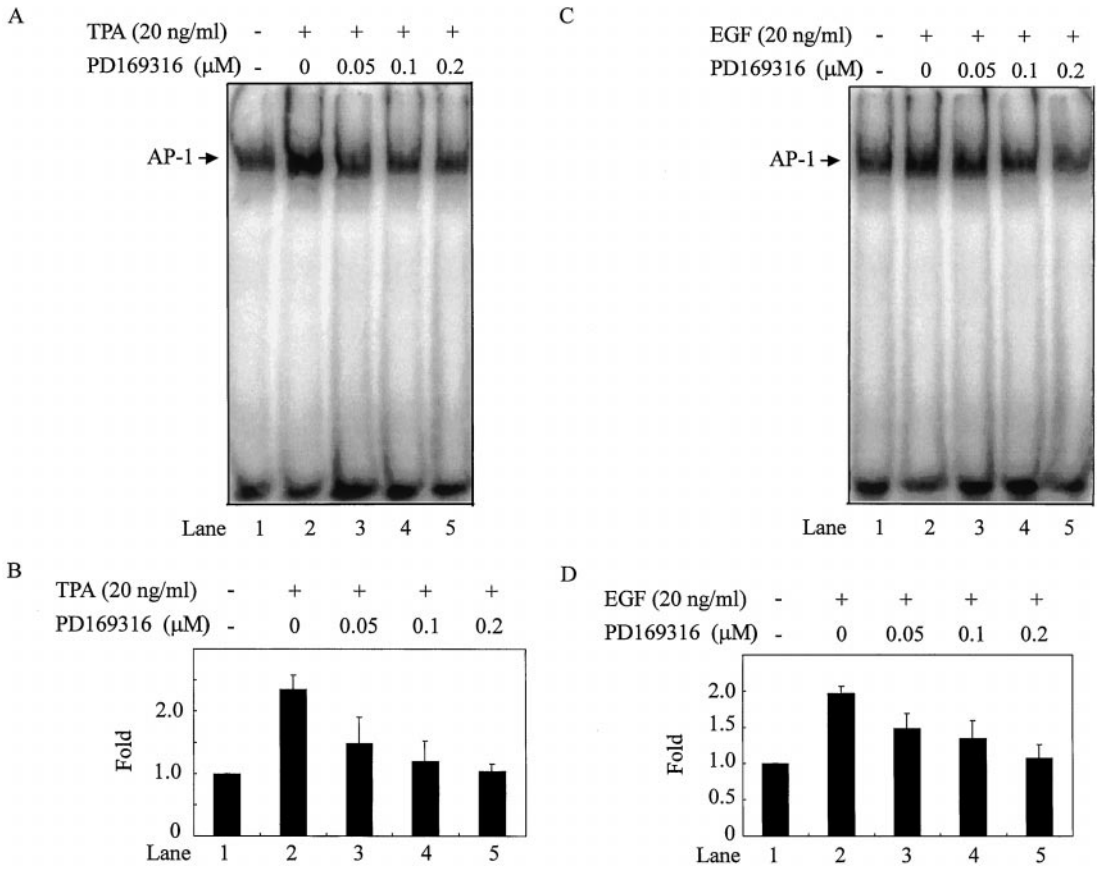

Fig. 9. PD169316 blocks TPA- or EGF-induced AP-1 DNA binding. JB6 Cl 41 cells were treated, nuclear proteins were extracted, and EMSA was carried out as described in "Materials and Methods." In *A*, PD169316 in the concentration indicated effectively blocks TPA-induced AP-1 DNA binding (*Lanes 3–5* compared with *Lane 2*). *B*, quantitation analysis of Fig. 9*A* from three performances of EMSA using nuclear extracts from different cell preparations. In *C*, PD169316 in the concentration indicated effectively blocks EGF-induced AP-1 DNA binding (*Lanes 3–5* compared with *Lane 2*). *D*, quantitation analysis of Fig. 9*C* from three performances of EMSA using nuclear extracts from different cell preparations.

revealed that NB11 has an antimutagenic function (33). In the current study, we investigated the effects of these two noni glycosides on AP-1 transactivation and subsequent cell transformation in the mouse epidermal JB6 cell line. The JB6 cell line is a well-established system used extensively as an *in vitro* model for the study of tumor promotion and antitumor promotion (12–14, 34). Our results indicated that both compounds blocked TPA- or EGF-induced JNK activity, which most likely explains their inhibitory effect on AP-1 transactivation and the subsequent reduction in cell transformation induced by TPA or EGF.

As a sequence-specific transcriptional activator, AP-1 mediates a broad range of external stimuli that lead to gene transcription. Many stimuli, including TPA, growth factors, and UV radiation that induce AP-1, are associated with tumorigenesis (5, 6). Neoplastic transformation is often associated with a dramatic increase in AP-1 activity (35), and this transient induction of AP-1 has been shown to be involved in the promotion of epidermal tumors (17, 36–38). Constitutive AP-1 activity has been associated with the malignant conversion of papillomas to carcinomas (39) as well. Chemopreventive agents or modification of AP-1 proteins that inhibit AP-1 activation are effective in preventing cell transformation or tumorigenesis (18, 35, 40, 41). Both TPA and EGF are tumor promoters that stimulate malignant transformation. In our experiments, both NB10 and NB11 suppressed TPA- or EGF-stimulated JB6 cell transformation on soft agar. A corresponding inhibition of TPA- or EGF-induced AP-1 activity was also found, suggesting that the inhibition of tumorigenesis by these compounds is through the inhibition of AP-1 activity.

Many mechanisms are involved in the up- and down-regulation of AP-1 activity (5). MAPKs are the most common pathways known to mediate AP-1 function (27), and in the current experiment, TPA or EGF activated all three members of the MAPK family: JNKs, ERKs, and p38 kinases. However, NB10 and NB11 were only able to block TPA- or EGF-stimulated JNK activity and not ERK or p38 kinase activities. Although both JNKs and ERKs of the MAPK family have been reported to be able to induce AP-1 activity (27), each of the kinases may activate different AP-1 components resulting in the transcription of different genes (27). Many reports indicated that JNKs are critical in mediating AP-1 transactivation and malignant transformation (19, 42–45). For instance, we have reported that TNF-α-induced cell transformation requires activation of JNKs (46). We also reported recently that TPA-induced skin tumorigenesis was strikingly suppressed in JNK-2-deficient mice (47). In the human lung carcinoma A549 cell line, when EGF-induced JNK activity was blocked, the EGF-stimulated proliferation effect, but not the basal proliferation rate, was completely blocked (42). Moreover, PD98059, a specific MAP/ERK inhibitor (48), completely blocked ERK activation by EGF and basal cell growth, but not EGF-stimulated growth, indicating that JNKs may be a preferential effector pathway for the growth-inducing properties of EGF (42). In our experiments, the inhibition of TPA- or EGF-induced JNK activity by NB10 or NB11 agreed well with the inhibitory effects of the compounds on TPA- or EGF-induced AP-1 activity, AP-1 DNA binding, and cell transformation. Furthermore, PD169316, a specific inhibitor of both MAPK p38 and JNKs (30, 31), also suppressed TPA- or EGF-induced AP-1 DNA binding and cell transformation. Considering the fact that MAPK families JNKs and ERKs, but not p38, are mainly involved in activating AP-1 (27), it was more likely that PD169316 suppressed AP-1 via its blocking of JNKs.

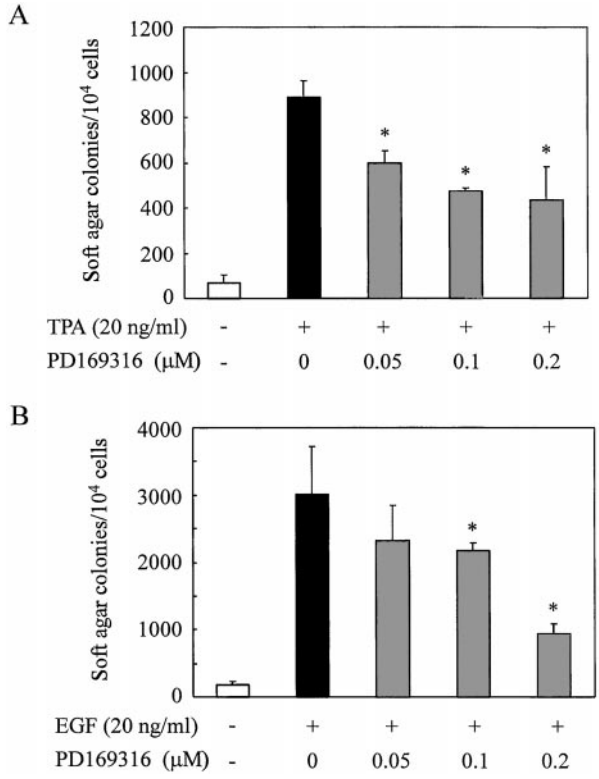

Fig. 10. PD169316 suppresses both TPA- and EGF-stimulated cell transformation on soft agar. JB6 Cl 41 cells seeded in triplicate wells were exposed to 20 ng/ml TPA or EGF with or without PD169316 at the concentrations indicated and as described in "Materials and Methods." Cell colonies were scored after 4 weeks of incubation in a 37°C, 5% CO_2 atmosphere. In *A,* PD169316 effectively inhibited TPA-induced cell transformation on soft agar [*, a significant ($P < 0.01$) inhibition compared with TPA treatment with no inhibitor present; *bars,* SD of three wells each concentration]. In *B,* PD169316 inhibited EGF-induced cell transformation on soft agar [*, a significant ($P < 0.01$) inhibition compared with EGF treatment with no inhibitor present; *bars,* SD of three wells each concentration].

These results indicated that the inhibition of JNKs was critical in diminishing TPA- or EGF-induced AP-1 activity and subsequent cell transformation.

The two glycosides, the novel NB10 and the known NB11 from *Morinda Citrifolia* (noni), were effective in inhibiting cell transformation induced by TPA or EGF in the mouse epidermal JB6 cell line. The inhibition was found to be associated with the inhibitory effects of these compounds on AP-1 activity. The compounds also blocked phosphorylation of c-Jun, a substrate of JNKs, suggesting that JNKs are a critical target for the compounds in mediating AP-1 activity and cell transformation.

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