Title:
Anthocyanidins inhibit activator protein 1 activity and cell transformation: Structure-activity relationship and molecular mechanisms

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Running Title: Inhibition of AP-1 activity and cell transformation by anthocyanidins
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Abbreviations: AP-1, activator protein-1; DMSO, Dimethyl sulfoxide; ERK, extracellular signal-regulated kinase; EMEN, Eagle’s minimum essential medium; FBS, fetal bovine serum; INT, p-iodonitrotetrazolium violet; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; P+, promotion sensitive; RO, reactive oxygen; SAPK, stress-activated protein kinase; SEK, SAPK/ERK kinase; SOD, superoxide dismutase; TPA, 12-O-tetradecanoylphorbol-13-acetate; TRE, TPA-response elements.

Anthocyanins are the chemical components that give the intense color to many fruits and vegetables, such as blueberries, red cabbages and purple sweet potatoes. Extensive studies have indicated that anthocyanins have strong antioxidant activities. To investigate the mechanism of anthocyanidins as an anti-cancer food source, six kinds of anthocyanidins representing the aglycons of most anthocyanins, were used to examine their effects on tumor promotion in mouse JB6 cells, a validated model for screening cancer chemopreventive agents and elucidating the molecular mechanisms. Of the six anthocyanins tested, only those with an ortho-dihydroxyphenyl structure on the B-ring suppressed 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced cell transformation and AP-1 transactivation, suggesting that the ortho-dihydroxyphenyl may contribute to the inhibitory action. Delphinidin, but not peonidin, blocked the phosphorylation of protein kinases in the extracellular signal-regulated protein kinase (ERK) pathway at early times and the c-Jun NH-terminal kinase (JNK) signaling pathway at later times. p38 kinase was not inhibited by delphinidin. Furthermore, two MAPK specific inhibitors (SP600125 for JNK and UO126 for ERK) could specifically block the activation of JNK and ERK and cell transformation. Those results demonstrate that...
Anthocyanidins contribute to the inhibition of tumorigenesis by blocking activation of the MAPK pathway. These findings provide the first molecular basis for the anticarcinogenic action of anthocyanidins.

**Introduction**

Anthocyanins are widely spread in colored fruits and vegetables such as berries, red grapes, purple sweet potatoes, and red cabbages (1, 2). Depending on their pH and the presence of chelating metal ions, they are intensely colored in blue, violet, or red. Anthocyanins are naturally present as glycosides having glucose, galactose, rhamnose, xylose or arabinose attached to the aglycon (named anthocyanidin) nucleus, and number several hundred depending on the glycoside structures (1). On the other hand, anthocyanidins are limited to a few structure variants, including delphinidin, cyanidin, petunidin, pelargonidin, peonidin and malvidin (Fig.1) representing the aglycons of most anthocyanins in plants. Depending on the nutrition customs, the daily intake of anthocyanins in humans has been estimated to range from several milligrams to hundreds of milligrams. The American diet can have much as 180-215 mg/day (3). An enhanced intake of anthocyanin is increasing because extracts with high anthocyanin content from bilberry or elderberry are commercially available. In addition, anthocyanins can be directly absorbed and distributed to the blood (4-6). Epidemiological investigations have indicated that the moderate consumption of anthocyanins through the intake of the products such as red wine (7) or bilberry extract (8) is associated with a lower risk of coronary heart disease. Extensive studies indicate that anthocyanins have strong free radical scavenging and antioxidant activities (9-13), those have been suggested to play an important role in prevention against mutagenesis and carcinogenesis (14). Anthocyanins also show inhibitory effects on the growth of some cancer cells (15-19). Recent study reported (20) that oral intake of anthocyanins from purple sweet potato color and red cabbage color suppressed rat colon carcinogenesis induced by 1,2-dimethylhydrazine (DMH) and 2-amino-1-methyl-6-phenylimidazo [4,5-b] pyridine (PhIP). However, the molecular mechanism is unknown. Thus, the possibility and mechanisms for anthocyanin application in anticarcinogenesis need to be considered at molecular level.

Carcinogenesis is a multistage process that encompasses three independent steps: initiation, promotion, and progression (21, 22). During these steps, a number of critical gene regulation events occur. Thus, understanding the molecular basis of carcinogenesis is important for prevention of carcinogenesis. Recent studies have suggested that the transcription factor activator protein 1 (AP-1) plays an important role in promoting carcinogenesis (23, 24). AP-1 is a dimeric protein typically composed of the products of the *jun* and *fos* oncogene families (24). AP-1 dimers bind to the promoter regions on DNA that contain 12-0-tetradecanoylphorbol-13-acetate (TPA) response elements (TRE) to activate the transcription of genes involved in cell proliferation (24), transformation (25, 26), and apoptosis (27). A variety of stimuli, such as phorbol esters (25, 2648), UV radiation (29), growth factors, (30) and oxidative agents (31), can stimulate AP-1 activity by activating mitogen-activated protein kinases (MAPK), such as extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase or stress activated protein kinase (JNK/SAPK), and p38 kinase. Increased AP-1 activity has been shown to be involved in the tumor promotion and progression of various types of cancers, such as skin (32, 33), lung (34) and breast cancer (35). *In vivo* mouse data also demonstrate that AP-1 activity is required for tumor promotion (3657).
JB6 mouse epidermal cells provide a cell culture based model for studying tumor promotion (23). Observations first recorded in the JB6 model have been validated in other culture models, including human keratinocytes (38, 39), as well as in vivo models using transgenic mice (36). In mouse epidermal cells, tumor promoters such as TPA, EGF and TNF-alpha induce AP-1 activity and neoplastic transformation in promotion-sensitive (P⁺), but not promotion-resistant (P⁻) JB6 cell lines by activating MAPK including ERK, JNK or p38 kinase (25, 26, 53). The induced AP-1 activity and neoplastic transformation can be blocked by chemopreventive agents such as retinoids (25, 40), pyrrolidine dithiocarbamate (41), tea polyphenols (42, 43), and glycoside compounds (44). Many of these inhibitors have been shown to be active not only in the JB6 transformation model but also in mouse skin tumor promotion in vivo. Thus, mouse epidermal JB6 cells provide a validated model to screen cancer chemopreventive agents, and to elucidate their mechanisms at the molecular level.

Given the epidemiological data suggesting cancer prevention properties of anthocyanin and the role of AP-1 activity in carcinogenesis, we tested the effects of six anthocyanidins, which represent the aglycons of the most abundant anthocyanins in plants, on TPA-induced AP-1 activity and cell transformation in mouse JB6 cells. Our results demonstrate the possible mechanism of the inhibitory actions of anthocyanidins on tumor promotion. We show that the ortho-dihydroxyphenyl on the B-ring is critical for the prevention of tumor promotion. Finally, active anthocyanidins appear to block activation of ERK and JNK signaling pathways leading to activation of AP-1.

**Materials and methods**

*Reagents and cell culture*

Delphinidin chloride, cyanidin chloride, pelargonidin chloride, peonidin chloride and malvidin chloride purified by HPLC were obtained from Extrasynthese (Genay, France), and their purity are 99.7, 98.7, 99.9, 99.4 and 95.4%, respectively. Petunidin chloride was prepared by acid hydrolysis of petanin (petunidin 3-p-coumaroylrutinoside-5-glucoside) isolated from purple potato (45), and purified by HPLC (purity is over 95%). All of anthocyanidins used were dissolved in dimethyl sulfoxide (DMSO, final concentration was 0.2%). Antibodies were from Cell Signaling Technology (Beverly, MA). Luciferase assay substrate was obtained from Promega (Madison, WI). Fetal bovine serum (FBS) was from Equitech-Bio (Kerrville, TX). TPA, p-iodonitrotetrazolium violet (INT), superoxide dismutase (SOD), D-mannitol and catalase were from Sigma (St. Louis, MO).

The JB6 P⁺ mouse epidermal cell line, Cl41 (28), and its AP-1-luciferase reporter stable transfectant P⁺11 cells (40) were cultured at 37°C, 5% CO₂ in EMEM containing 5% FBS, 2 mM L-glutamine and 25 µg gentamicin.

*Anchorage-independent transformation assay*

The inhibitory effects of anthocyanidins on TPA-induced cell transformation were investigated in the parental JB6 Cl41 cells or AP-1-luciferase stable transfectant JB6 cells (P⁺11) (46). There is no marked difference in TPA-induced transformation between both cell lines (Hou et al., unpublished data). Cells (1 × 10⁶) were suspended in 2 ml of 0.38% BME agar medium over 3 ml of 0.5% BME agar medium containing 10% FBS, 20 ng/ml TPA with or without anthocyanidins (5 µM to 20 µM). The cultures were maintained in a 37°C, 5% CO₂ incubator for 14 days, and the anchorage-independent colonies after staining with INT were scored by a computerized image analyzer. The efficiency of anthocyanidin inhibition of TPA-induced cell transformation is expressed...
as a percentage of the transformation frequency when the cells were treated with TPA alone.

**Luciferase assay for AP-1-dependent transactivation**

AP-1-luciferase stable transfectant JB6 cells (P+11) (46) were used to assay AP-1-dependent transactivation. Viable cells (2×10^4) were plated in a 48-well dish for 24 h before each experiment. The cells were starved by being cultured in 0.1% FBS-EMEM for another 18 h to eliminate the influence of FBS on AP-1 activity, and then treated with or without anthocyanidin for 30 min before they were exposed to 20 ng/ml TPA for 24 h. For antioxidant agents, the cells were treated with SOD, catalase or D-mannitol alone or with SOD plus anthocyanidins for 30 min, respectively, before the cells were exposed to 20 ng/ml TPA for 24 h. The cells were extracted with lysis buffer, and the luciferase activity was measured by a luminometer (Berthold) according to the supplier’s recommendations. AP-1 activity is expressed as fold induction relative to the control cells without TPA treatment (47, 48).

**Western blotting analysis**

After the cells (1.5×10^6) were cultured in 10-cm dish for 24 h, the cells were starved in serum-free for another 4 h to eliminate the influence of FBS on MAPK activation. The cells were then treated with or without delphinidin for 30 min before they were exposed to 20 ng/ml TPA for the different times. The harvested cells were lysed and the supernatants were boiled for 5 min. Protein concentration was determined using dye-binding protein assay kit (Bio-Rad) as described in manufacture’s manual. Lysate protein of 40 µg was run on 10% SDA-PAGE and electrophoretically transferred to PVDF membrane (Bio-Rad). After blotting, the membrane was incubated with specific primary antibody overnight at 4°C, and further incubated for 1 h with HRP-conjugated secondary antibody. Bound antibodies were detected by ECL system with a Lumino Image Analyzer. The relative amount of proteins associated with specific antibody was quantified by using the Imager Gauge Software (Fuji Photo Film).

**Statistical analyses**

Difference between the treated and the control was analyzed by Student’s t-test. A probability of P<0.05 was considered significant. The multiplicative models for the interaction between two agents were used to determine the effect of a combination of SOD with delphinidin (49, 50). The additive model predicts the effect of a combination to be equal to the effect of its constituents; an observed effect of the combination higher than predicted by the additive model indicates synergism (49).

**Results**

**Anthocyanidins inhibit TPA-induced JB6 cell transformation and AP-1 activation in a structure-activity relationship**

Cells (1×10^6) were exposed to 20 ng/ml TPA in soft agar for 14 days, 1000-2000 transformed colonies were induced whereas in the solvent control group (<0.1% DMSO) there is no colony formation. TPA-induced cell transformation was significantly inhibited by delphinidin, petunidin and cyanidin, but not by pelargonidin, peonidin and malvidin (P<0.05) at the concentration range from 5-20 µM. Previous studies have suggested that AP-1 transactivation is required for TPA-induced cell transformation in mouse JB6 cells (25, 40, 51). We thus tested the effects of those anthocyanidins on TPA-induced AP-1 activity by using a reporter gene assay. The
treatments of delphinidin, petunidin and cyanidin, but not pelargonidin, peonidin and malvidin, markedly inhibited TPA-induced AP-1 activity at the same concentration range (Fig. 2B; P<0.05). The inhibitory actions by delphinidin, petunidin and cyanidin were not caused by their cytotoxicity, because the concentration range that inhibited cell transformation and AP-1 activity did not affect cellular viability as measured by MTT assay (data not shown). These results indicate that inhibition of AP-1 activity by delphinidin, petunidin and cyanidin may be important in their inhibitory action against TPA-induced cell transformation.

The ortho-dihydroxyphenyl structure on the B-ring of anthocyanidins (Fig.1) may be essential for the inhibitory action because pelargonidin, peonidin and malvidin, having no such ortho-dihydroxyphenyl structure, failed to show the inhibitory effects.

**Delphinidin blocks TPA-induced ERK and JNK phosphorylation but not p38 phosphorylation**

Because delphinidin showed the strongest potent inhibition in AP-1 transactivation, we used delphinidin to further investigate the effects on MAPK pathways. MAPK pathways including ERK, JNK and p38 kinase influence AP-1 transactivation by increasing the abundance of AP-1 components and/or altering the phosphorylation of their subunits (52). Previous studies indicated that ERK activation is earlier event occurring at 0.5 h - 4 h following TPA treatments, while activation of JNK and p38 was a later event occurring at 6 h - 24 h in JB6 cells (53, Hou et al., unpublished data). Thus, we chose the time at 2 h to target ERK, and at 12 h to JNK and p38. As shown in Fig. 3A, TPA induced markedly phosphorylation of ERK at 2 h and phosphorylation of JNK and p38 at 12 h (lane 1). Delphinidin suppressed TPA-induced phosphorylation of ERK and JNK, respectively, in a dose-dependent manner (lane 2-6). However, the phosphorylation of p38 was not blocked by delphinidin. The results suggest that the mechanism by which delphinidin inhibits AP-1 transactivation may involve early inhibition of ERK phosphorylation and later inhibition of JNK phosphorylation, but does not involve targeting p38 kinase. As a control, we also examined the effect of peonidin, which showed no inhibition on cell transformation (Fig. 2A) and AP-1 activity (Fig. 2B), on MAPK. The data showed that peonidin did not block TPA-induced phosphorylation of JNK, ERK and p38 kinase (Fig. 3B). These results further supported that the suppression of MAPK activation by anthocyanidins were associated with their inhibitory effect on AP-1 activation and cell transformation.

**Delphinidin blocks the activation of JNK and ERK signaling cascades**

MAPK/ERK kinase (MEK) is a protein kinase that phosphorylates and activates ERK (54-56). To test whether delphinidin inhibits MEK phosphorylation, we examined the effect of delphinidin on the phosphorylation of MEK. As shown in Fig. 4A, delphinidin repressed TPA-induced MEK1/2 phosphorylation at the same time- and dose-range that blocked TPA-induced ERK phosphorylation. On the other hand, SAPK/ERK kinase 1(SEK1) is another protein kinase that phosphorylates and activates JNK (57-59). Thus, we tested the effect of delphinidin on the phosphorylation of SEK1 by a dose-response experiment (Fig. 4B). Delphinidin blocked TPA-induced SEK1 phosphorylation at the dose range that blocked TPA-induced JNK phosphorylation. Moreover, we also tested the effect of delphinidin on the phosphorylation of c-Jun, which is a JNK target. c-Jun phosphorylation was markedly blocked by delphinidin at the same concentration range while the total amount of c-Jun protein was not changed (Fig. 4C). Thus, it appears that the inhibition of AP-1 activity by delphinidin is mediated by blocking ERK and JNK signaling cascades.
UO126 and SP600125 suppress the activation of JNK and ERK and cell transformation
Our data indicate that delphinidin might inhibit AP-1 transactivation and cell transformation by an early inhibition of ERK activation and later inhibition of JNK activation. To more clearly identify which factor is more critical for blocking JB6 transformation, we used two specific inhibitors (SP600125 for JNK and UO126 for MEK1/2) to challenge to block TPA-induced MAPK activation and cell transformation. It is known that UO126 is a selective MEK1/2 inhibitor that inhibits the phosphorylation of ERK1/2 (60), and SP600125 is a selective JNK inhibitor that inhibits the phosphorylation of c-Jun (61). As showing in Fig. 5, both inhibitors could effectively block TPA-induced phosphorylation of ERK and c-Jun (Fig. 5A) and cell transformation (Fig. 5B), respectively. We could not distinguish which is more critical for blocking cell transformation although the inhibitory effect by UO126 appeared a little strong, but not significant (P>0.05), than that by SP600125 (Fig. 5B). These results indicate that both ERK and JNK cascades are critical in mediating cell transformation.

Synergy between delphinidin and SOD in inhibiting AP-1 activity
Several reports suggest that TPA treatment in JB6 cells is associated with the generation of ROS that further promotes neoplastic transformation (62, 63). To identify which species of TPA-induced RO is required for AP-1 activation in JB6 cells, three kinds of RO eliminating enzymes or agents including SOD (a scavenger of superoxide anion), catalase (a decomposer of hydrogen peroxide) and D-mannitol (a scavenger of hydroxyl radical) (62) were used. In the concentration range of the agents that did not affect cellular viability as measured by MTT assay (data not shown), TPA-induced AP-1 activity was significantly blocked by SOD, but not by catalase or D-mannitol (Fig. 6A; P<0.05). The results indicate that TPA-induced superoxide anion may contribute to AP-1 activation. Because either anthocyanidin (Fig. 2B) or SOD alone could inhibit AP-1 activity, we next treated the cells with combinations of the two agents. A greater inhibition was observed in combinations of SOD with delphinidin, cyanidin and petunidin, but not with pelargonidin, peonidin and malvidin (Fig. 6B), suggesting that the ortho-dihydroxyphenyl structure on the B-ring of anthocyanidins may be essential for this action. To distinguish whether the combination inhibition is additive or synergistic, the multiplicative model (49, 50) was applied to estimate the effect of combinations of SOD with delphinidin, a greatest inhibitor, at varying concentrations (Fig. 6C). The results obtained with 200 U/ml SOD indicate the actual AP-1 activity observed is less than that expected from additive effects calculated according to the multiplicative model (Fig. 6D). Similar results were also obtained with 10 and 100 U/ml SOD (data not shown). Thus, these results suggest that delphinidin and SOD appear to act in a synergistic manner to inhibit TPA-induced AP-1 activity.

Discussion
Anthocyanidins, aglycons of anthocyanins, are known to exist in red fruits and vegetables. This paper reports a molecular evidence for anticarcinogenesis by anthocyanidins. Our findings suggest that anthocyanidins inhibit TPA-induced MAPK, AP-1 activation and cell transformation. The ortho-dihydroxyphenyl structure on the B-ring of anthocyanin aglycon appears essential for the inhibitory action.
To elucidate the molecular events of anthocyanidins in the inhibition of AP-1 activity and cell transformation, we used delphinidin, which showed the strongest potent inhibition, to investigate the effects on signaling pathways mediating AP-1 activity.
Although many mechanisms may be involved in the up- and down-regulation of AP-1 activity, MAPK including ERK, JNK and p38 kinases are known to be common signaling pathways mediating AP-1 activity (24, 52). Our data indicate that delphinidin blocked ERK phosphorylation at early times and JNK phosphorylation at later times, but not p38 phosphorylation at any time (Fig.3). Moreover, we investigated the inhibition of delphinidin in the up- and down-pathway of MAPK, and found that the phosphorylation of MEK (an ERK kinase), SEK (a JNK kinase) and c-Jun (a phosphorylation target of ERK and JNK) were blocked in delphinidin-treated cells, too. These data suggest that the inhibition of TPA-induced AP-1 activity by delphinidin may involve the blockage of activation of ERK and JNK signaling cascades. Many previous reports indicate that ERK and/or JNK are able to induce AP-1 activity and cell transformation (26, 54, 64, 65). Some chemopreventive agents including tea polyphenols (42, 43) and glycoside compounds (44) inhibit AP-1 transactivation by blocking JNK activation. Recent studies have suggested that ERK also play a critical role in TPA-induced AP-1 activity and cell transformation in JB6 cell lines (26, 66, 65).

For instance, stable expression of dominant negative ERK in tumor promoter-sensitive cell line JB6 CI41 blocks TPA-induced cell transformation (66). In the present study, delphinidin blocked the TPA-induced activation of both ERK and JNK in different time fashion. Furthermore, two specific inhibitors (UO126 for MEK1/2 and SP600125 for JNK) could effectively block TPA-induced phosphorylation of ERK and c-Jun, and cell transformation at the same dose, respectively. Consistent with this finding, our data indicate that the inhibition of both ERK and JNK cascades are critical in diminishing TPA-induced cell transformation. Moreover, both ERK and JNK cascades were blocked by delphinidin, suggesting more upstream effectors may sever as the target(s) of delphinidin. Accumulated evidence has indicated that the upstream effector of ERK cascade is Ras/Raf, and that of JNK cascade is Rac/Rho (67, 68). Of which, MEKK1/3 may sever as a crosstalking between the two cascades for stress response (68). We are interested to clarify the upstream target(s) of the anthocyanidins in next study.

Another important question is how do the potent anthocyanidins block TPA-induced AP-1 activation. TPA treatment in JB6 might generate ROS that further promotes neoplastic transformation (64, 65). TPA-induced cell transformation in soft agar could be 90% blocked by the addition of SOD, but not by catalase or GSH-Px (60). In the present study, TPA-induced AP-1 activation was blocked by SOD, but not by catalase and mannitol, implicating superoxide anion in the process of activating AP-1. This requirement of superoxide for AP-1 activation has also been shown to occur in transgenic mice in vivo during skin tumor promotion (69). Recent report has indicated that anthocyanidins can also scavenge superoxide radicals when they encountered the radicals in an in vitro ESR system (70). The specific scavenging activity (ID=50) for superoxide radicals was, respectively, 2.2, 22, and 456 µM of delphinidin, cyanidin and pelargonidin (70). Thus, we investigate the effect of combinations of SOD with anthocyanins. A greater inhibition was observed in combinations of SOD with anthocyanins those have the ortho-dihydroxyphenyl structure on the B-ring of anthocyanidins (Fig. 5B), suggesting that this structure may contribute to the inhibition action. Furthermore, the results from multiplicative model analysis suggested that this greater inhibition between SOD and delphinidin is synergistic, not additive. Our findings together with other reports suggest that the inhibitory effects of anthocyanidins on AP-1 activation are due in part to their potent scavenging activity for superoxide radicals and in part to blocking MAPK. Both targets may be important in the cancer prevention activity of anthocyanidins although the mechanistic relations between them are needed to clarify. It is noteworthy that delphinidin inhibits AP-1 activity by
blocking ERK at early times and JNK activation at later times in the present study. SOD selectively inhibits the TPA-induced activation of protein kinase Epsilon and prevents subsequent activation of JNK2 in response to TPA, thereby delaying AP-1 activation and inhibiting mouse skin tumor promotion (69). Thus, the signaling pathways blocked by delphinidin or SOD may differ in part.

It has been reported that anthocyanins have strong free radical scavenging and antioxidant activities (9-13), those have been suggested to play an important role in prevention against mutagenesis and carcinogenesis. Moreover, oral intake of anthocyanins from purple sweet potato color and red cabbage color could suppress rat colon carcinogenesis induced by DMH and PhIP (20). Thus, our data together with other reports suggest a possibility that moderate consumption of anthocyanins through the intake of the products such as bilberry extracts may be linked with cancer prevention. Further epidemiological study on this aspect is required.

In summary, we have provided evidence for a novel mechanism of the anti-tumor promotion action by six anthocyanidins. Of which, only those having an ortho-dihydroxyphenyl structure on the B-ring of aglycon suppressed TPA-induced cell transformation and AP-1 transactivation. The potent anthocyanidins may block TPA-induced ERK and JNK signaling cascades leading to activation of AP-1. These findings provide the first molecular basis for the anticarcinogenic action of anthocyanins.

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References


Legends to figures

Figure 1. Chemical structure of anthocyanidins.

Figure 2. Anthocyanidins inhibit TPA-induced JB6 cell transformation (A) and AP-1 activation (B) in a structure-activity relationship. For cell transformation, JB6 Cl41 cells (1 × 10⁴) were exposed to 20 ng/ml TPA with or without the indicated concentrations of anthocyanidins on soft agar medium. The cell colonies were scored by a computerized image analyzer after a 14-day incubation in a 37°C, 5% CO₂ incubator. The efficiency of anthocyanidin inhibition of cell transformation is expressed as a percentage of the transformation frequency when the cells were treated with TPA alone. For report gene assay, AP-1 luciferase reporter plasmid stable transfectant JB P⁺11 cells (2 × 10⁵) were seeded into each well of 48-well plates. The cells were treated with or without the indicated concentrations of anthocyanidins for 30 min before they were exposed to 20 ng/ml TPA for 24 h. The luciferase activity was assayed, and AP-1 activity is expressed as fold induction to the control cells without TPA treatment. Each value represents the mean ± SD of 4-5 separate experiments. *P<0.05 vs. control.

Figure 3. Delphinidin blocks TPA-induced ERK and JNK, but not p38 phosphorylation. (A) Delphinidin blocks TPA-induced ERK and JNK phosphorylation. After JB6 cells were starved in serum-free medium for 4 h. The cells were treated with the indicated concentrations of delphinidin for 30 min, and then exposed to 20 ng/ml TPA for 2h to target ERK, and for 12h to target JNK and p38. (B) Peonidin does not block TPA-induced phosphorylation of ERK, JNK and p38. After JB6 cells were starved in serum-free medium for 4 h. The cells were treated with 20 µM peonidin or without for 30 min, and then exposed to 20 ng/ml TPA for 2h to target ERK, and for 12h to target JNK and p38. Western blotting analysis of MAPKs were performed with specific antibodies, respectively, as described in “Materials and Methods”. Histograms show the densitometric analysis of phosphorylated protein expression normalized to total MAPK. The data represents the mean ± SD of 3-4 separate experiments, and the figure is a representative of those experiments each with similar results.

Figure 4. Delphinidin blocks TPA-induced phosphorylation of MEK1/2 (A), SEK1 (B) and c-Jun (C). Cell treatment and Western blotting analysis were done as described in Fig. 3. Histograms show the densitometric analysis of phosphorylated protein expression normalized to total MAPK. The data represents the mean ± SD of 3-4 separate experiments, and the figure is a representative of those experiments each with similar results.

Figure 5. UO126 and SP600125 suppress phosphorylation of ERK and c-Jun (A), and cell transformation (B). Cell treatment and Western blotting analysis were done as described in Fig. 3. In brief, the cells were treated with UO126 or SP600125 (10 µM) for 30 min, and then exposed to 20 ng/ml TPA for 2h or 12 h, respectively. Histograms show the densitometric analysis of phosphorylated protein expression normalized to total MAPK. The data represents the mean ± SD of 3-4 separate experiments, and the figure is a representative of those experiments each with similar results. For cell transformation, JB6 Cl41 cells (1 × 10⁴) were exposed to 20 ng/ml TPA with or without the indicated concentrations of UO126 or SP600125 on soft agar medium. The inhibitory efficiency of UO126 or SP600125 on cell transformation was estimated as described in Fig. 2A. *P<0.05 vs. control.
Figure 6. Synergistic inhibition between delphinidin and SOD. JB P+11 cell culture and luciferase activity assay were done as described in Fig. 2B. Each value represents the mean ± SD of 3-6 separate experiments. (A) SOD, but not catalase or D-mannitol, inhibits TPA-induced AP-1 activity. The cells were treated with or without SOD, catalase and D-mannitol for 30 min at the indicated concentrations before they were exposed to 20 ng/ml TPA for 24 h. *P<0.05 vs. control. (B) Delphinidin, cyanidin and petunidin, but not pelargonidin, peonidin or malvidin, show greater inhibition of TPA-induced AP-1 activity with SOD. The cells were treated by six anthocyanidins (5 µM) with or without SOD (200 U/ml) for 30 min before they were exposed to 20 ng/ml TPA for 24 h. *P<0.05 vs. SOD or anthocyanidin alone. (C) Delphinidin synergistically inhibits AP-1 activity with SOD. The cells were treated with the indicated concentrations of delphinidin in the presence or absence of various doses of SOD. Results represent the mean ± SD of three experiments as a percentage of AP-1 activity in the presence of TPA alone. (D) Comparison between the actual AP-1 activity (shaded bars) and the expected value for additive effects (open bars) in combinations of them.
Fig. 1. Hou et al.

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**B**

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**C**

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Fig. 4. Hou et al.
A

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**P-c-Jun**

**c-Jun**

**Relative density of P-JNK (fold)**

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**U0126 (µM)**

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**P-ERK**

**ERK**

**Relative density of P-ERK (fold)**

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**Fig. 5. Hou et al.**
Fig. 5. Hou et al.
Fig. 6. Hou et al.
Fig. 6. Hou et al.