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Cyanidin suppresses ultraviolet B-induced COX-2 expression in epidermal cells by targeting MKK4, MEK1, and Raf-1

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ABSTRACT

Skin cancer is the most frequently diagnosed cancer in the United States. Ultraviolet B (UVB) rays (wavelength: 280–320 nm) play a pivotal role in the development of skin cancer by inducing the expression of inflammatory proteins such as cyclooxygenase-2 (COX-2). Cyanidin, the most plentiful of the plant pigments known as anthocyanidins, is a potent chemopreventive agent. In the present study, we examined the molecular mechanisms underlying the chemopreventive activity of cyanidin and identified its molecular targets. Cyanidin inhibited UVB-induced COX-2 expression and prostaglandin E_2 secretion in the epidermal skin cell line JB6 P+ by suppressing the transactivation of nuclear factor-κB and activator protein-1 which are well-known transcription factors regulated by mitogen-activated protein kinase. Cyanidin markedly inhibited the phosphorylation of JNK1/2, ERK1/2, and MEK1/2 than the of MKK4 and Raf-1, two upstream kinases of JNK1/2, ERK1/2, and MEK1/2. Cyanidin significantly suppressed the activities of MKK4, MEK1, and Raf-1 through direct binding. Transient transfection of a small interfering RNA specific for MKK4 inhibited the UVB-induced expression of COX-2 in JB6 P+ cells, as did the expression of a dominant-negative ERK2 mutant. We conclude that MKK4, MEK1, and Raf-1 are targets of cyanidin for the suppression of UVB-induced COX-2 expression.

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1. Introduction

Ultraviolet (UV) light (wavelength: 10–400 nm; energy: 3–124 eV) is a major cause of all types of skin cancer, the most frequently diagnosed cancer in the Unites States [1]. Solar UVC (200–280 nm) rays are completely absorbed by the Earth's ozone layer and atmospheric oxygen. The ozone layer also absorbs most UVB (280–320 nm) rays; however, some UVB penetrates the Earth's atmosphere. UVA (320–400 nm) rays are not absorbed by ozone. As a result, UV light at the Earth's surface contains 98% UVA and 2% UVB. Because UVB rays are 1000 times more effective at causing sunburn than UVA rays, UVB rays are the primary form of electromagnetic radiation that induces skin diseases, including cancer. Thus, efforts to prevent skin cancer have focused primarily on inhibiting the effects of UVB rays [2].

Cyclooxygenase (COX) is the rate-limiting enzyme in the conversion of arachidonic acid to prostaglandins [3]. One major isoform, COX-1, is stably expressed in most tissues, whereas COX-2, the second major isoform, is expressed only during inflammation. Many lines of evidence suggest that COX-2 is related to carcinogenesis. For example, several types of cancer cells over-express COX-2 [4], and COX-2 increases invasiveness and decreases adhesion in epithelial cells, increases cellular resistance to apoptosis and angiogenesis, and modulates host immune surveillance [5]. Further, the expression of COX-2 in skin can be induced by UVB irradiation [6], and treatment with specific COX-2 inhibitors or the knockout of the COX-2 gene have been reported to reduce UVB-induced skin carcinogenesis in mice. Thus, COX-2 is an excellent target for the prevention of UVB-induced skin cancer [7].

Mitogen-activated protein kinase (MAPK) signaling pathways play important roles in many biological processes, including inflammation, apoptosis, proliferation, and differentiation. The MAPKs include extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs), and p38. These kinases are activated by UVB exposure and by specific upstream kinases such as Raf and MAPK/ERK kinase (MEK)1/2, which activate ERKs, MAPK kinase (MKK)4/7, which activates JNKs, and MKK3/6, which activates p38

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[8]. Because MAPKs are the primary mediators of UVB-induced COX-2 expression [9], the inhibition of enzymes in the MAPK pathways reduces COX-2 expression and is a powerful strategy for preventing the harmful effects of UVB rays.

The plant pigments known as anthocyanidins are found in grapes, apples, berries, purple cabbage, and corn. Average consumption in the United States is 180–215 mg/day [10]. Previous studies have shown that anthocyanidins have various health benefits, such as anti-inflammatory and anti-tumorigenic effects, but the most abundant anthocyanidin, cyanidin has not been studied as thoroughly as some other anthocyanidins or cyanidin glucosides. Although some researchers have reported that cyanidin has anti-carcinogenic or antiatherosclerotic effects [11–13], the molecular mechanisms underlying the effects of cyanidin and its molecular targets have not been explored. In the present study, we investigated these molecular mechanisms and targets and found that cyanidin suppresses UVB-induced COX-2 expression by acting as a potent inhibitor of MKK4, MEK1, and Raf-1.

2. Materials and methods

2.1. Chemicals

Cyanidin (>96% pure) was purchased from Extrasynthese (Genay Cedex, France) and dissolved in dimethyl sulfoxide (Sigma–Aldrich, St. Louis, MO) to produce a 4 mM stock solution. The stock solution was stored in 40 μL aliquots at −70 °C and used as soon as possible after preparation. Eagle's minimum essential medium (MEM), and fetal bovine serum (FBS) were obtained from Gibco BRL (Carlsbad, CA). Antibodies against phosphorylated MEK1/2 (Ser217/221), phosphorylated Raf-1 (Ser338), phosphorylated MKK4 (Ser257/Thr261), phosphorylated JNK1/2 (Thr183/Tyr185), total JNK1/2, phosphorylated c-Jun (Ser63), total c-Jun, phosphorylated $p90^{RSK}$ (Thr359/Ser363), and total $p90^{RSK}$ were purchased from Cell Signaling Technology (Beverly, MA). Antibodies against total MEK1/2, total Raf-1, total MKK4, phosphorylated ERKs (Thr202/Tyr204), and total ERK were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-β-actin was purchased from Sigma–Aldrich, while CNBr–Sepharose 4B, and [γ-^{32}P]ATP were obtained from GE Healthcare (Piscataway, NJ, USA). G418 and the luciferase assay substrate were purchased from Promega (Madison, WI, USA).

2.2. Cell culture

JB6 P+ mouse epidermal cells (JB6 P+ cells) obtained form The American Type Culture Collection (Rockville, MD) were cultured in monolayers in 5% FBS in MEM (5% FBS/MEM) containing 1000 units of penicillin and 1 mg/mL of streptomycin at 37 °C under 5% CO_2. JB6 P+ cells stably transfected with an AP-1 or NF-κB promoter–luciferase reporter plasmid or with a plasmid encoding a dominant-negative ERK2 mutant were a gift from Dr. Zigang Dong [14]. The cells were maintained in 5% FBS/MEM containing 200 μg/mL G418.

2.3. UVB irradiation

A UVB irradiation system with a spectral peak at 312 nm (Bio-Link Crosslinker, Vilber Lourmat, France) was used to stimulate JB6 P+ cells growing in serum-free media. This UVB machine emitted about 70% of UVB and 30% UVA irradiation. Cells were exposed to UVB at a dose of 0.5 kJ/m^2 and then cultured for various lengths of time as indicated.

2.4. Western blot analysis

JB6 P+ cells were cultured for 48 h, incubated in 0.1% FBS/MEM for an additional 24 h, treated with or without cyanidin for 1 h, and exposed to 0.5 kJ/m^2 UVB. At various intervals after UVB exposure, the cells were scraped, treated with lysis buffer [10 mM Tris (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 10% glycerol, and 1× protease inhibitor cocktail] for 40 min on ice, and then centrifuged at 16,000 × g for 10 min. The protein concentration of the supernatant fraction was measured using a dye-binding protein assay kit as described by the manufacturer (Bio-Rad Laboratories, Hercules, CA). Lysate aliquots containing 40 μg of protein were subjected to 10% SDS-polyacrylamide gel electrophoresis (PAGE) and then transferred to a polyvinylidene fluoride membrane (Millipore Corporation, Bedford, MA). After transfer, the membrane was blocked in 5% fat-free milk for 1 h and incubated with a specific primary antibody for 2 h at room temperature. After incubation with a horseradish peroxidase-conjugated secondary antibody, protein bands were detected using an enhanced chemiluminescence detection kit (GE Healthcare).

2.5. Luciferase assays for COX-2, AP-1, and NF-κB transcriptional activity

Confluent monolayers of JB6 P+ cells stably transfected with a COX-2, AP-1, or NF-κB promoter–luciferase reporter plasmid were trypsinized, harvested, and suspended in 100 μL of 5% FBS/MEM. Each well of a 96-well plate was seeded with 8 × 10^3 cells, and the plates were incubated at 37 °C in a 5%-CO_2 incubator until the cells reached 80–90% confluence. The cells were then cultured in 0.1% FBS/MEM for 24 h, treated with various concentrations of cyanidin for 1 h, exposed to 0.5 kJ/m^2 UVB, and harvested after 6 h. The cells were disrupted with 100 μL of luciferase lysis buffer [0.1 M potassium phosphate buffer (pH 7.8), 1% Triton X-100, 1 mM DTT, and 2 mM EDTA)], and COX-2, AP-1, or NF-κB promoter activity was determined by measuring luciferase activity with a luminometer (Microlumat Plus LB 96 V; Berthold Technologies, Bad Wildbach, Germany).

2.6. Prostaglandin E_2 (PGE_2) assay

Two milliliters of JB6 P+ cells in growth medium were plated into each well of a six-well plate. The cells were then grown to 80% confluence, treated with cyanidin for 1 h, exposed to 0.5 kJ/m^2 UVB, and harvested 18 h later. The amount of PGE_2 released into the medium was measured using a PGE_2 enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI).

2.7. In vitro kinase assay

The levels of activity of MKK4, MEK1, and Raf-1 were determined using assay kits obtained from Upstate Biotechnology (Lake Placid, NY) according to the instructions provided by the manufacturer. Briefly, each reaction contained recombinant MKK4, MEK1, or Raf-1 protein (10 ng) in 20 μL of assay dilution buffer [20 mM MOPS (pH 7.2), 25 mM β-glycerolphosphate, 5 mM EGTA, 1 mM Na_3VO_4, and 1 mM DTT]. The protein was incubated with cyanidin (0, 5, 10, or 20 μM) in a magnesium–ATP cocktail buffer for 10 min at room temperature. The MKK4 assay also contained 250 ng of inactive JNK1; the MEK1 assay also contained 1 μg of inactive ERK2 substrate peptide, while the Raf-1 assay also contained 0.4 μg of inactive MEK substrate peptide and 1 μg of inactive ERK2 substrate peptide. A 4 μL aliquot was removed after incubation of the reaction mixture for 30 min at 30 °C; thereafter, 20 μg of the MBP substrate peptide (Raf-1 and MEK1) and 300 μg of ATF-2 (MKK4) peptide were added. A diluted [γ-^{32}P]ATP solution (10 μL) was added to each reaction, which was then incubated for 10 min at 30 °C. Reaction aliquots (15 μL) were transferred to p81 filter paper, washed three times with 0.75% phosphoric acid for 5 min each, and washed with acetone

for 2 min. The incorporated radioactivity was measured using a scintillation counter. Each experiment was performed three times.

2.8. Ex vivo immunoprecipitation and kinase assays in JB6 P+ cells

For *ex vivo* MKK4, MEK1, and Raf-1 kinase assays, JB6 P+ cells were grown to 80% confluence and then serum-starved in 0.1% FBS/MEM for 24 h at 37 °C. The starved cells were then treated with 0–20 μM cyanidin for 1 h, exposed to 0.5 kJ/m^{2}UVB, and harvested after 15 min. They were then disrupted with lysis buffer [20 mM Tris–HCl (pH 7.4), 1 mM EDTA, 150 mM NaCl, 1 mM EGTA, 1% Triton X-100, 1 mM β-glycerophosphate, 1 mg/mL leupeptin, 1 mM Na_3VO_4, and 1 mM PMSF] and centrifuged at 16,000 × *g* for 10 min. Lysate samples containing 500 μg of protein were added to immunoprecipitation reaction mixtures containing an antibody against MKK4, MEK1, or Raf-1 and incubated at 4 °C overnight. Protein A/G Plus agarose beads were added to the reactions, and the resulting mixtures were rotated continuously for another 3 h at 4 °C. The beads were washed three times with kinase buffer [20 mM MOPS (pH 7.2) and 25 mM β-glycerophosphate]. Radioactive incorporation was determined as for the *in vitro* kinase assay described above. Data are presented as the mean of data points from three independent experiments.

2.9. Direct and cell-based pull-down assays

To couple cyanidin to Sepharose 4B beads, freeze-dried CNBr Sepharose 4B powder (0.3 g) was suspended in 5 mL of 1 mM HCl, mixed with cyanidin (1 mg) in 5 mL of coupling solution [0.1 M $NaHCO_3$ (pH 8.3) and 0.5 M NaCl], and incubated at 4 °C overnight with end-over-end rotation. The beads were then transferred to 0.1 M Tris–HCl buffer (pH 8.0) and incubated at 4 °C overnight with rotation as above. After three washes with 0.1 M acetate buffer (pH 4.0)/0.5 M NaCl and one wash with 0.1 M Tris–HCl (pH 8.0)/0.5 M NaCl, the cyanidin-conjugated Sepharose 4B beads (100 μL of a 50% slurry) were mixed with recombinant MKK4, MEK1, or Raf-1 protein (0.2 μg) or a JB6 P+ cellular supernatant fraction (500 μg) in pull-down buffer [50 mM Tris (pH 7.5), 5 mM EDTA, 150 mM NaCl, 1 mM DTT, 0.01% Nonidet P-40, and 0.02 mM PMSF]supplemented with 2 μg/mL bovine serum albumin and 1× protease inhibitor mixture(A non-conjugated Sepharose 4B slurry was used as a negative control). After incubation with gentle rocking overnight at 4 °C, the beads were washed five times with pull-down buffer and the proteins bound to the beads were analyzed by Western blotting.

2.10. siRNA-mediated silencing of the MKK4 gene

Three small interfering RNAs (siRNAs) designed to knock down murine MKK4 gene expression and a control siRNA that contains a scrambled sequence that does not lead to the specific degradation of any known cellular mRNA, were purchased from Bioneer (Daejun, Korea). JB6 P+ were transfected with siRNA using INTERFERin^{TM} (Polyplus-Transfection, Illkirch, France). The final siRNA concentration was 10 nM

2.11. ATP–cyanidin competition assay

Recombinant MKK4, MEK1, or Raf-1 (0.2 μg) was incubated with cyanidin (0, 5, 10, or 20 μM) and ATP–agarose 4B beads in reaction buffer for 2 h at 4 °C. After washing, the proteins bound to

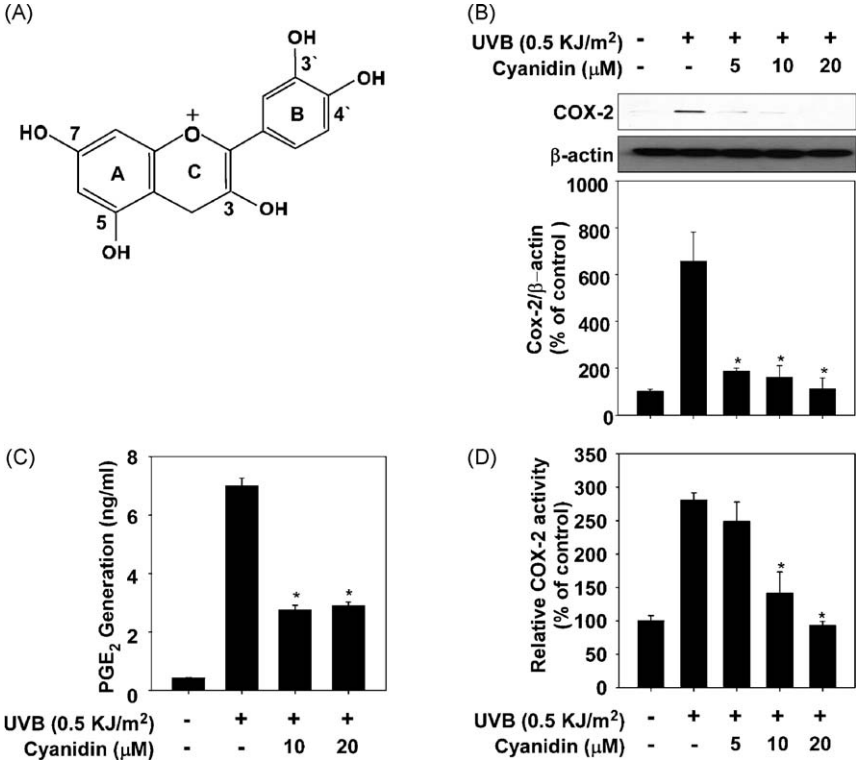

Fig. 1. Cyanidin inhibits UVB-induced COX-2 expression and PGE_2 production in JB6 P+ cells. (A) Chemical structure of cyanidin. (B) Cyanidin inhibits UVB-induced COX-2 expression. JB6 P+ cells were seeded, grown for two days, and then starved for 24 h in medium containing 0.1% FBS. They were then treated with cyanidin at the indicated concentration (0, 5, 10, or 20 μM) for 1 h, exposed to 0.5 kJ/m^2 UVB, and harvested 4 h later. COX-2 expression was determined by Western blot analysis as described in Section 2. β-actin was used as an internal control to verify equal protein loading. The COX-2 and β-actin bands were quantified by densitometric analysis using Image J software. The data shown are representative of three independent experiments. (C) Cyanidin inhibits UVB-induced PGE_2 production. JB6 P+ cells were grown, serum-starved, and UVB-treated as in (B) then harvested 18 h later. PGE_2 production was measured using a PGE_2 assay kit as described in Section 2. (D) Cyanidin inhibits UVB-induced COX-2 promoter activity. JB6 P+ cells stably transfected with a COX-2 promoter–luciferase reporter plasmid were grown, serum-starved, and UVB-treated as in (B) then harvested 6 h later. Relative luciferase activity was determined as described in Section 2. In (B–D), statistical significance was calculated by the one-way ANOVA test with a Dunnett's post-test ($^*P < 0.01$) in comparison with UVB-treated control.

the ATP–agarose 4B beads were resolved by SDS-PAGE and analyzed by Western blotting using specific antibodies.

2.12. Molecular modeling

The docking of cyanidin onto MKK4 or Raf-1 was examined using model structures generated by Geno3D (http://geno3d-pbil.ibcp.fr) using the published crystal coordinates of MKK7 (PDB accession code 2DYL) and B-raf (1UWH), respectively, as homologous templates. The docking of cyanidin onto MEK1 was examined using published MEK1 coordinates (1S9J). Insight II (Accelrys Inc., San Diego, CA, USA) was used for the modeling and structural analyses.

2.13. Statistical analysis

The results are expressed as mean ± SD of the indicated number of independent experiments, and statistical analysis was performed using the one-way ANOVA test, with a Dunnett's post-test. A difference with P-value < 0.01 was considered statistically significant.

3. Results

3.1. Cyanidin inhibits the UVB-induced expression of COX-2 and production of PGE_2 in JB6 P+ cells

First we investigated the effects of cyanidin on the UVB-induced expression of COX-2 in JB6 P+ cells. UVB exposure upregulated COX-2 expression and that cyanidin inhibited this upregulation (Fig. 1B). In addition, UVB exposure increased the level of PGE_2, a primary product of COX-2, and cyanidin suppressed this increase (Fig. 1C). To determine whether cyanidin regulates COX-2 expression at the level of transcription, we used a reporter gene assay. JB6 P+ cells stably transfected with a COX-2 promoter–luciferase reporter plasmid were treated with various concentrations of cyanidin, exposed to UVB rays, and assayed for luciferase reporter activity. The resulting data showed inhibition of UVB-induced COX-2 promoter activity by cyanidin (Fig. 1D), indicating that cyanidin suppresses the transcriptional activation of COX-2 by UVB in JB6 P+ cells.

3.2. Cyanidin attenuates the UVB-induced transactivation of AP-1 and NF-κB in JB6 P+ cells

Since AP-1 and NF-κB are major transcriptional regulators of COX-2 expression, we next measured the effects of cyanidin on the UVB-induced transactivation of AP-1 (Fig. 2A) and NF-κB (Fig. 2B) using JB6 P+ cells stably transfected with AP-1 or NF-κB promoter–luciferase reporter plasmids. Cyanidin inhibited the UVB-induced transactivation of AP-1 (Fig. 2A) and NF-κB (Fig. 2B) in a dose-dependent manner, suggesting that its ability to inhibit AP-1 and NF-κB transactivation may underlie its anti-inflammatory and anti-carcinogenic effects.

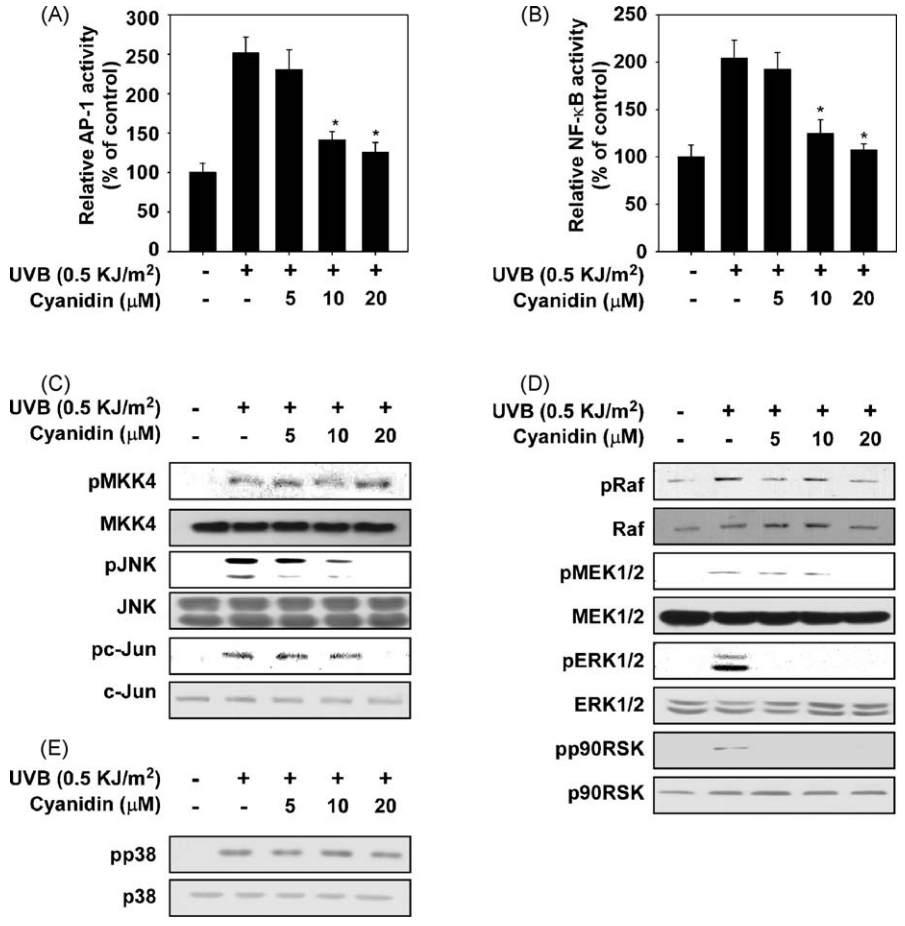

Fig. 2. Cyanidin inhibits UVB-induced AP-1 and NF-κB transactivation by suppressing the JNK1/2–c-Jun and MEK1/2–ERK1/2–$p90^{RSK}$ pathways. (A and B) Cyanidin inhibits the UVB-induced transactivation of AP-1 (A) and NF-κB (B). JB6 P+ cells stably transfected with an AP-1 or NF-κB promoter–luciferase reporter plasmid were treated with cyanidin at the indicated concentration (0, 5, 10, or 20 μM) for 1 h, exposed to 0.5 kJ/m^2 UVB, harvested 6 h later, and assayed for relative luciferase activity. The data shown represent means ± S.D. Statistical significance was calculated by the one-way ANOVA test with a Dunnett's post-test (*$P < 0.01$) in comparison with UVB-treated control. (C–E) JB6 P+ cells were treated with cyanidin at the indicated concentration (0, 10, or 20 μM) for 1 h, exposed to 0.5 kJ/m^2 UVB, harvested after 30 min, and analyzed by Western blotting using the indicated specific antibodies.

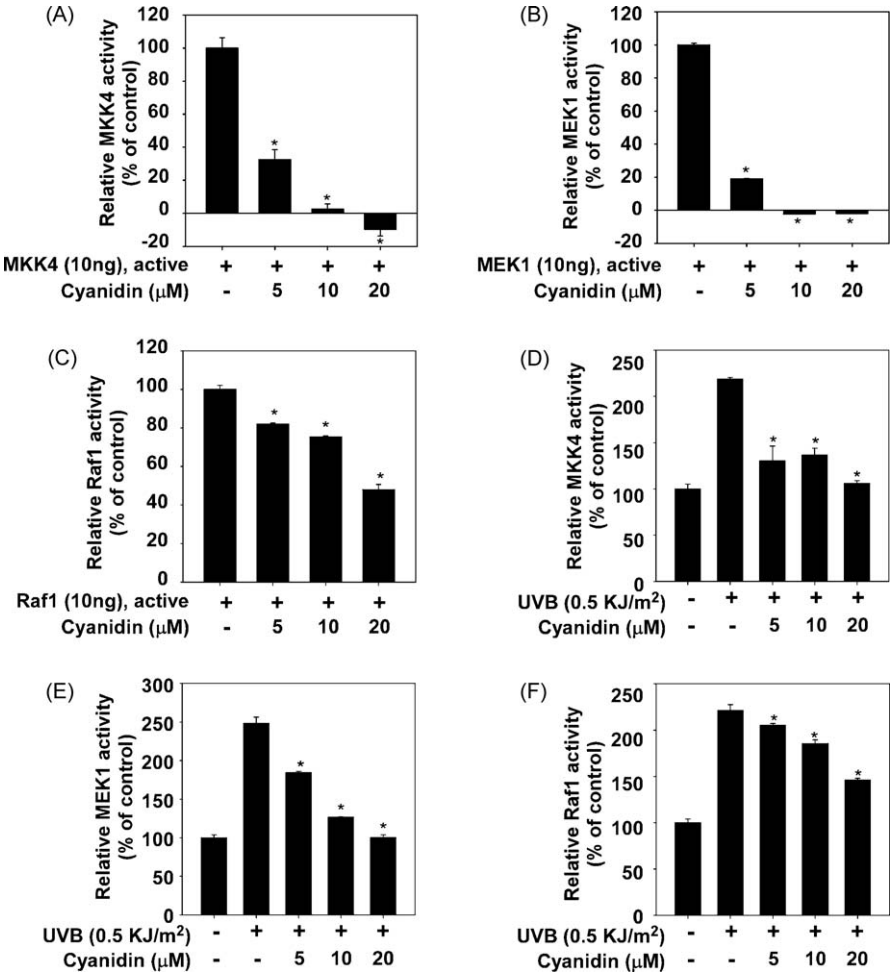

Fig. 3. Cyanidin inhibits MKK4, Raf-1, and MEK1 kinase activity *in vitro* and *ex vivo*. (A–C) The kinase activity of recombinant MKK4 (A), MEK1 (B), and Raf-1 (C) was determined *in vitro* as described in Section 2. The data shown represent means ± S.D. Statistical significance was calculated by the one-way ANOVA test with a Dunnett's post-test (*$P < 0.01$) in comparison with untreated control. (D–F) Cyanidin inhibits the induction of MKK4, Raf-1, and MEK1 kinase activity by UVB exposure. JB6 P+ cells were treated with cyanidin at the indicated concentration (0, 5, 10, or 20 μM) for 1 h, exposed to 0.5 kJ/m^2 UVB, and harvested 15 min later. The cellular levels of MKK4 (D), MEK1 (E), and Raf-1 (F) kinase activity were determined as described in Section 2. The data shown represent means ± S.D. Statistical significance was calculated by the one-way ANOVA test with a Dunnett's post-test (*$P < 0.01$) in comparison with UVB-treated control.

3.3. Cyanidin inhibits the induction of MKK4–JNK1/2–c-Jun and Raf-1–MEK1/2–ERK1/2 signaling by UVB

AP-1 and NF-κB are regulated by various signaling pathways, including MAPKs [15–16]. MAPKs phosphorylate and thereby activate AP-1 subunits such as c-Jun. The ERK1/2–p90^{RSK} pathway regulates NF-κB by phosphorylating IKKα [17] which is degraded by proteasomal processes after phosphorylation. NF-κB is also regulated by MAPKs through a variety of other routes [18–19]. When we tested cyanidin for its effects on UVB-induced MAPK signal transduction, we found that it suppressed the UVB-induced phosphorylation of JNK1/2 (Fig. 2C) and ERK1/2 (Fig. 2D). Surprisingly, the phosphorylation status of MKK4, a JNK1/2 kinase, was unchanged by cyanidin treatment. We were unable to detect UVB-induced phosphorylation of MKK7, another JNK1/2 kinase. Although cyanidin also decreased the phosphorylation of MEK1/2, ERK1/2, and Raf-1, the effect was greatest for ERK1/2 and weakest for Raf-1. These data indicate that cyanidin inhibits the phosphorylation of downstream kinases more strongly than it inhibits the phosphorylation of upstream kinases. The phosphorylation status of p38 did not change (Fig. 2E).

3.4. Cyanidin inhibits the kinase activities of MKK4, Raf-1, and MEK1

To confirm that cyanidin acts as a signaling inhibitor, we examined its ability to inhibit the kinase activities of MKK4, Raf-1, and MEK1. Cyanidin completely blocked the activities of MKK4 (Fig. 3A) and MEK1 (Fig. 3B) and partially blocked (by 50%) the activity of Raf-1 (Fig. 3C). These *in vitro* effects were mimicked in our cell-based system; cyanidin inhibited the UVB-mediated induction of these kinase activities in JB6 P+ cells (Fig. 3D–F).

3.5. Cyanidin binds directly to MKK4, MEK1, and Raf-1

To examine whether the inhibitory effect of cyanidin on MKK4, MEK1, and Raf-1 is due to direct physical interaction of cyanidin with the kinases, we performed *in vitro* chemical pull-down assays. Cyanidin bound recombinant MKK4, MEK1, and Raf-1 both *in vitro* (Fig. 4A) and ex vivo (Fig. 4B). Next, to examine the mode of cyanidin binding to MKK4, MEK1, and Raf-1, we performed ATP competition assays. ATP competed with cyanidin for MKK4 binding but not for MEK1 or Raf-1 binding (Fig. 4C). These results indicate that cyanidin binds to the ATP binding pocket of MKK4 and that it binds to Raf-1 and MEK1 at allosteric sites.

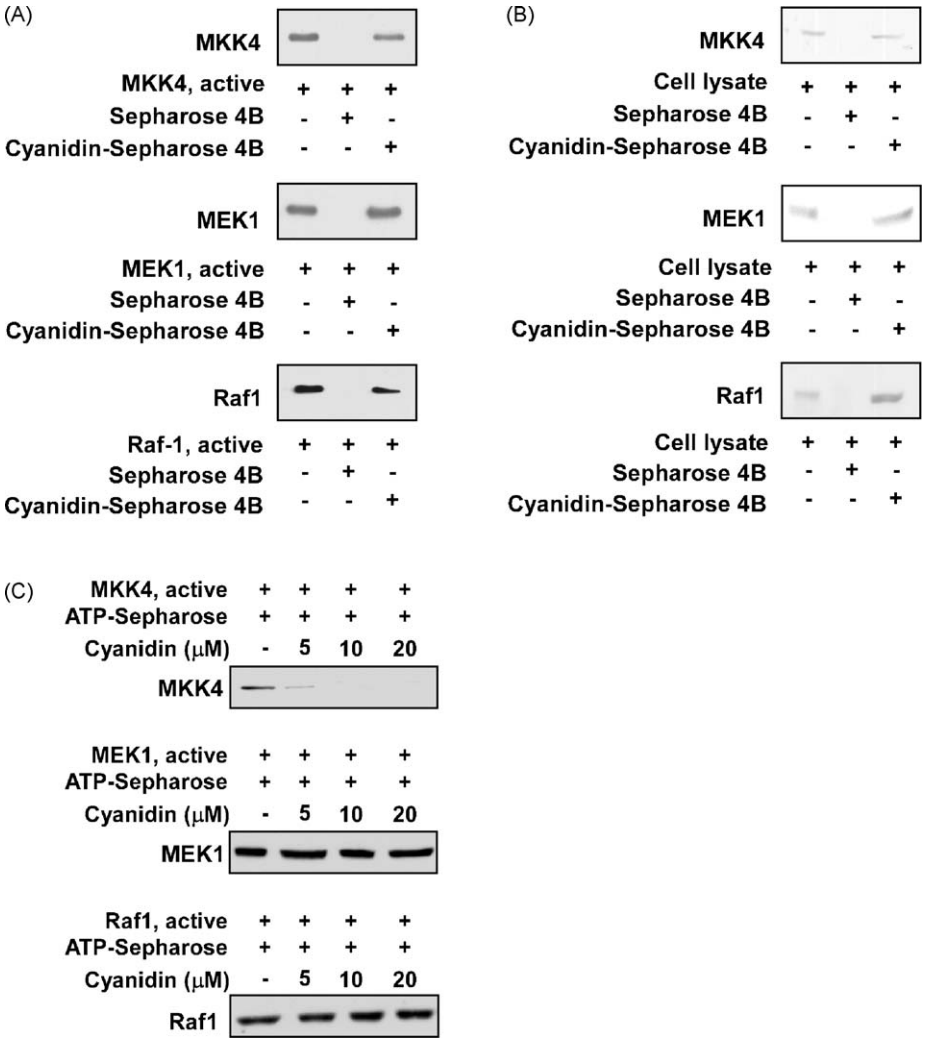

Fig. 4. Binding of cyanidin to MKK4, Raf-1, and MEK1 *in vitro* and *ex vivo*. (A) Cyanidin binds to recombinant MKK4 (upper panel), MEK1 (middle panel), and Raf-1 (lower panel) *in vitro*. Each recombinant protein was incubated with cyanidin–CNBr Sepharose 4B or CNBr Sepharose 4B beads (negative control) overnight at 4 °C. After the beads were washed, the proteins bound to the beads were analyzed by Western blotting. (B) Cyanidin binds MKK4 (upper panel), MEK1 (middle panel), and Raf-1 (lower panel) *ex vivo*. Cell lysates from UVB-treated JB6 P+ cells were incubated with cyanidin–CNBr Sepharose 4B or CNBr Sepharose 4B beads (negative control) overnight at 4 °C. After the beads were washed, the proteins bound to the beads were analyzed by Western blotting. (C) Cyanidin competes with ATP for binding to MKK4 (upper panel), but does not compete with ATP for binding to MEK1 (middle panel) or Raf-1 (lower panel). The recombinant proteins were incubated with cyanidin at the indicated concentration (0, 5, 10, or 20 μM) overnight at 4 °C. ATP–agarose beads were then added to the binding reactions, and the resulting mixtures were incubated for another 2 h. The beads were washed, and the amount of protein bound to the beads was analyzed by Western blotting.

3.6. The MKK4–JNK1/2 and Raf-1–MEK1/2–ERK1/2 pathways are required for UVB-induced COX-2 expression in JB6 P+ cells

To examine whether the MKK4–JNK1/2 and Raf-1–MEK1/2–ERK1/2 pathways are required for UVB-induced COX-2 expression in JB6 P+ cells, we used two approaches. First, we transiently transfected MKK4 siRNA into JB6 P+ cells. The resulting RNA interference suppressed MKK4 protein expression, inhibited UVB-induced COX-2 expression (Fig. 5A), and suppressed the UVB-induced phosphorylation of JNK1/2 but not of p38 (Fig. 5B). Second, we used JB6 P+ cells expressing a dominant-negative ERK2 mutant. In these cells, UVB exposure failed to induce the expression of COX-2 (Fig. 5C) or the phosphorylation of ERK2 (Fig. 5D).

4. Discussion

Cyanidin is an anthocyanidin found at high levels in many fruits, including berries, apples, and plums [20]. It has antioxidant effects [21], induces cell cycle arrest in several human cancer cell lines [22], and protects against DNA cleavage and free radical accumulation [23]. It also inhibits the PDGF(AB)-induced release of vascular endothelial growth factor in vascular smooth muscle cells [12] and tumor necrosis factor (TNF)-α-induced apoptosis in endothelial cells [11]. In the present study, we found that cyanidin inhibites UVB-induced expression of COX-2.

The transcription factor AP-1 is a dimer variously composed of c-Fos, c-Jun, ATF, and JDP subunits. It plays important roles in apoptosis, cellular proliferation, and neoplastic transformation. NF-κB is a major transcription factor that regulates both innate and adaptive immunity, and which participates in many inflammatory diseases, including cancer [24–25]. Both AP-1 and NF-κB are activated by extracellular stimuli, such as UVB rays, through MAPKs. The inhibition of AP-1 and NF-κB transactivation attenuates the expression of COX-2, a major inflammatory gene [26]. In this study, cyanidin inhibited UVB-induced COX-2 promoter activity and UVB-induced AP-1 and NF-κB transactivation. We conclude that cyanidin inhibits UVB-induced COX-2 expression by regulating two of its upstream signaling molecules, AP-1 and NF-κB.

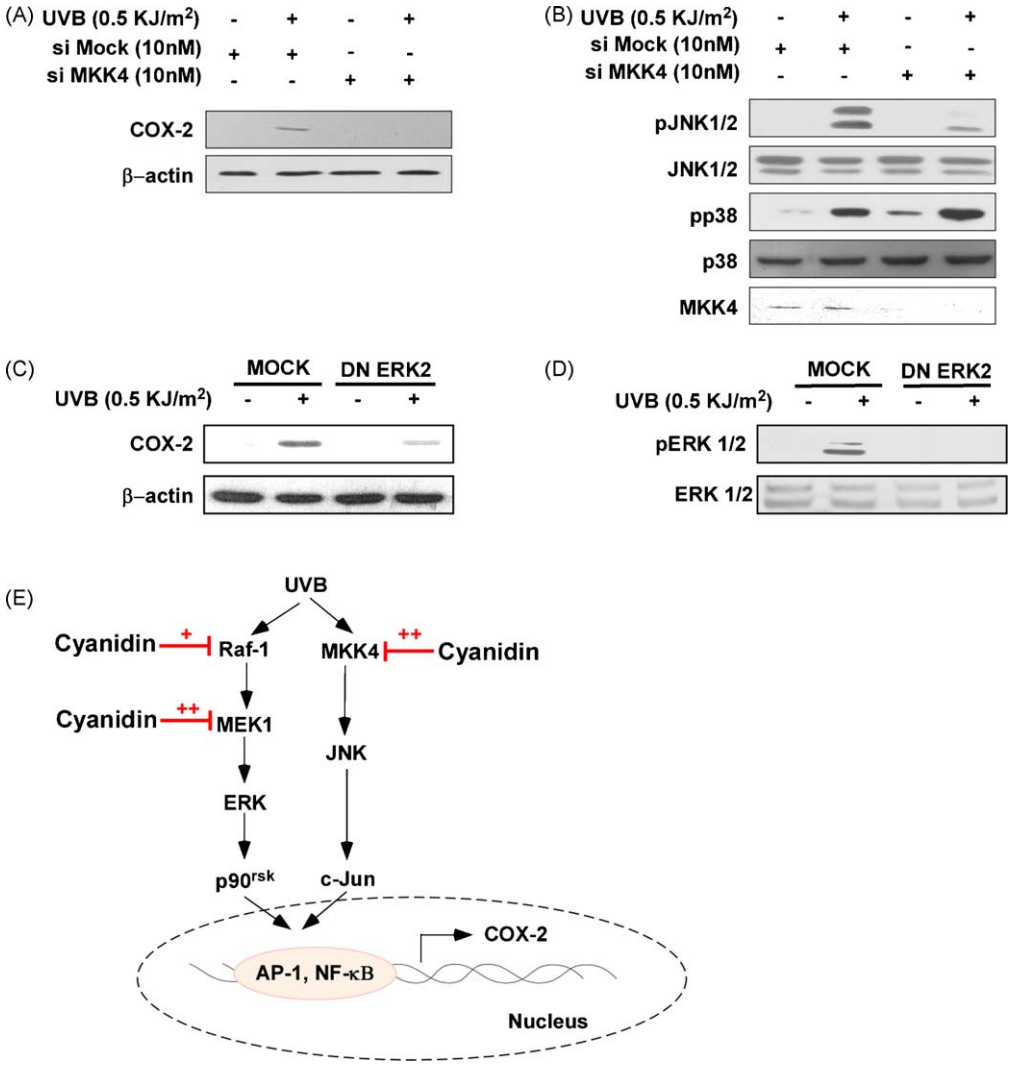

Fig. 5. Involvement of MKK4 and ERK2 in the UVB-induced upregulation of COX-2 in JB6 P+ cells. (A and B) MKK4 siRNA inhibits UVB-induced COX-2 expression. JB6 P+ cells were transfected with MKK4 siRNA as described in Section 2, exposed to 0.5 kJ/m^2 UVB, and harvested 4 h (A) or 15 min (B) later. The cells were disrupted, and the levels of COX-2 protein in the cell lysates were determined by Western blot analysis. (C and D) Dominant-negative (DN)-ERK2 suppresses UVB-induced COX-2 expression. JB6 P+ cells stably transfected with the DN-ERK2 plasmid were seeded, exposed to 0.5 kJ/m^2 UVB, and harvested 4 h (C) or 15 min (D) later. The cells were disrupted, and the levels of protein in the cell lysates were determined by Western blot analysis. (E) Simplified model of the effects of cyanidin on UVB-induced COX-2 expression.

The extensively studied MAPK pathways regulate AP-1 and NF-κB [9]. Because UVB rays activate ERK1/2, p38, and JNK1/2, the successful inhibition of at least one of these MAPKs may attenuate the harmful effects of UVB irradiation. In this study, we found that cyanidin inhibited the phosphorylation of JNK1/2 and ERK1/2, but not of p38. To elucidate the mechanism underlying these effects, we looked at the phosphorylation status of the upstream of JNK1/2 and ERK1/2. Cyanidin did not inhibit the UVB-induced phosphorylation of MKK4. Inhibition of Raf-1 phosphorylation is pretty weaker than that of MEK1/2 and ERK1/2. Many previous reports have suggested that the anti-inflammatory effects of natural compounds stem from their antioxidant activities [27–29] and are therefore nonspecific. However, our data show that cyanidin inhibits a specific event in MAPK signal transduction. Thus, we postulate that cyanidin acts as a specific signaling inhibitor rather than as a nonspecific chemical inhibitor.

MKK4 is a member of the MAPK kinase family. This kinase appears to phosphorylate p38 and JNK1/2 in response to specific stimuli in certain cell types [30]. Considerable attention has been paid to the putative role of MKK4 as a p38 or JNK1/2 kinase. One study reported that MKK4$^{-/-}$and MKK4$^{+/+}$ mouse embryonic fibroblasts (MEFs) exhibited no difference in phosphorylation status or p38 activity after stimulation with TNF-α and UV light, but that the phosphorylation of JNK1/2 was impaired in MKK4$^{-/-}$MEFs [31]. In the present study, we observed that treatment with an siRNA specific for MKK4 reduced the UVB-induced phosphorylation of JNK1/2, but not p38, in JB6 P+ cells, consistent with our cyanidin data showing reduced phosphorylation of JNK1/2, but not of p38, in UVB-stimulated JB6 P+ cells.

Because Raf-1 and MEK1/2 are well-known upstream kinases of ERK1/2, we examined the effect of cyanidin on Raf-1 and MEK1 activity. We found that cyanidin inhibited these kinases by binding to them. Therefore, we conclude that the inhibition of MKK4 activity by cyanidin results in the inhibition of JNK1/2 phosphorylation, and that the inhibition of MEK1 and Raf-1 activity by cyanidin results in the inhibition of ERK1/2 phosphorylation.

To determine whether the inhibition of MKK4 and ERK2 is involved in the suppression of COX-2 expression by cyanidin, we examined the effects of MKK4 siRNA and a dominant-negative ERK2 mutant on UVB-induced COX-2 expression. Both the siRNA and mutant ERK2 impaired the induction of COX-2 expression by UVB, showing that MKK4 and ERK1/2 are involved in UVB-induced

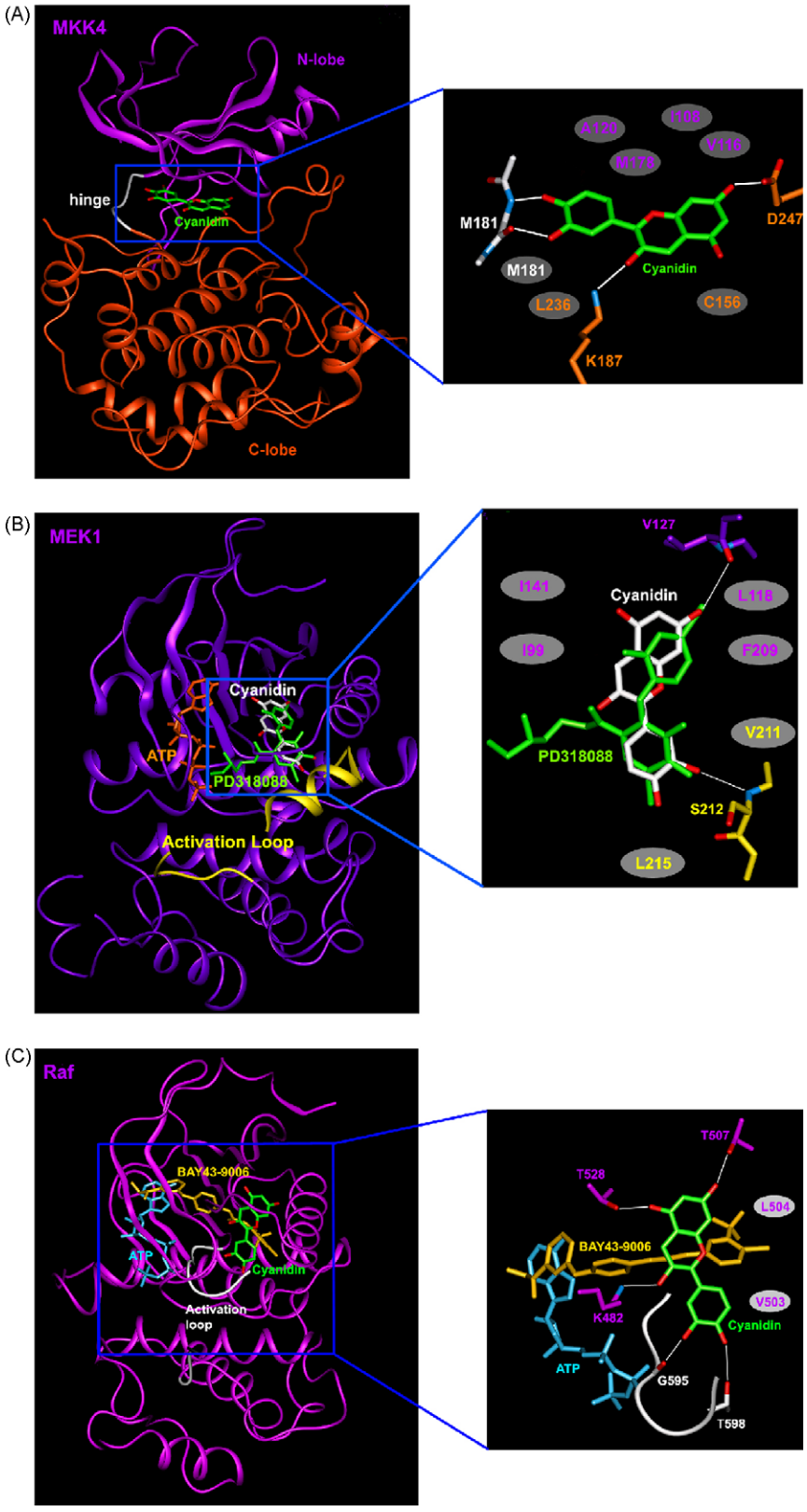

Fig. 6. Hypothetical structures of cyanidin co-complexes with MKK4, MEK1, and Raf-1. In close-up views of the inhibitor interactions, H-bonds are shown as white lines, while hydrophobic residues interacting with cyanidin are encircled by gray ellipses. (A) Cyanidin (green carbon atoms) binds to the ATP binding site in the kinase domain of MKK4. The hinge loop is colored white. (B) Cyanidin (white carbon atoms) binds to the pocket adjacent to the ATP binding site of MEK1. ATP is shown in orange. PD318088 (green) is superimposed on the model structure of the MEK1–cyanidin complex for comparison. The partially disordered activation loop is shown in yellow. (C) Cyanidin (green carbon atoms) binds to the pocket adjacent to the ATP binding site in Raf. ATP is shown in cyan. BAY43-9006 (yellow) is overlaid on the model structure. The partially disordered activation loop is shown in white (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article).

COX-2 expression in JB6 P+ cells. Therefore, cyanidin exerts its cancer chemopreventive effects by directly inhibiting the MKK4–JNK1/2 and Raf-1–MEK1/2–ERK1/2 pathways, leading to the subsequent suppression of UVB-induced COX-2 expression.

To further investigate the molecular basis for MKK4, MEK1, and Raf-1 inhibition by cyanidin, we also carried out docking studies. Because no crystal structure of MKK4 is available, a model structure for the MKK4 kinase domain was derived from the crystal structure of MKK7, which is 66% homologous to MKK4 in its amino acid sequence. The kinase domain of MKK4 consists of an N-lobe and C-lobe linked through a loop known as the "hinge region." The backbone of this loop forms hydrogen bonds (H-bonds) with the adenine moiety of ATP. Because our experimental results indicated that cyanidin is an ATP-competitive inhibitor of MKK4, we attempted to dock cyanidin onto the ATP binding site of MKK4. The cyanidin structure docked readily onto the ATP binding site of MKK4 (Fig. 6A), forming H-bonds with the backbone of the hinge region. This mode of binding is similar to that of other ATP-competitive kinase inhibitors. The hydroxyl groups at positions 3′ and 4′ of cyanidin form H-bonds with the backbone atoms of Met181, while the hydroxyl groups at positions 3 and 7 form H-bonds with the side chains of Lys187 and Asp247, respectively. The inhibitor is sandwiched by the side chains of the hydrophobic residues in the ATP binding site, including those of Ala120, Met178, Ile108, Val116, Cys156, Leu236, and Met181.

In our modeling study of MEK1, we used the crystal structure of MEK1 complexed with PD318088, a non-ATP-competitive inhibitor of MEK1 [32]. Like PD318088, cyanidin docked onto a pocket separate form but adjacent to the ATP binding site (Fig. 6B). This result is consistent with our finding that cyanidin does not compete with ATP for MEK1 binding. In its docked position, cyanidin forms an H-bond between its hydroxyl group at position 7 and the backbone carbonyl group of Val127, a residue in the binding pocket. In addition, the hydrophobic surface formed by Ile99, Ile141, Phe209, and Leu118 makes several van der Waals interactions with cyanidin. Cyanidin also interacts with residues in the MEK1 activation loop in its inactive conformation; it forms van der Waals interactions with Val211 and Leu215 and an H-bond between the 3′-hydroxyl group and backbone amide group of Ser212. These interactions of cyanidin with the activation loop are expected to lock MEK1 into a catalytically inactive state by stabilizing its inactive conformation.

We carried out a docking study for Raf-1 using the crystal structure of B-raf, which is highly homologous to Raf-1 (80% identical in terms of its primary sequence) since no crystal structure of Raf-1 is available. In the crystal structure of B-raf in complex with its ATP-competitive inhibitor BAY43-9006, the inhibitor occupies the adenine binding site and stabilizes the inactive conformation of the activation loop by interacting with the phenyl ring of Phe594 [33]. However, because our experiments showed that cyanidin does not compete with ATP for Raf-1 binding, we did not attempt to dock cyanidin onto the ATP binding site of B-raf. In the hypothetical structure of the ternary B-raf/ATP/cyanidin complex, cyanidin docks onto a pocket separate from but adjacent to the ATP binding site of B-raf (Fig. 6C). The hydroxyl groups at positions 3, 5, and 7 form H-bonds with the side chains of Lys482, Thr528, and Thr507, respectively. Cyanidin also makes contacts with Leu504 and Val503 through hydrophobic interactions. The 3′- and 4′-hydroxyl groups of cyanidin form H-bonds with the backbone carbonyl group of Gly595 and the side chain of Thr598, thereby holding the activation loop of B-raf in an inactive conformation. In contrast, BAY43-9006 stabilizes the inactive conformation only through hydrophobic interactions. Additional X-ray crystallographic studies to determine the inhibitor co-complex structures should elucidate the exact binding mode of cyanidin to MKK4, MEK1, and Raf-1.

Our recent studies have shown that flavonoids have healthful effects that stem from their ability to directly target multiple-kinases and inhibit the core signaling pathways of disease, such as MAPK pathways [34–36]. After the success of the prescription drug Gleevec, pharmaceutical researchers have paid significant attention to the potential of multiple-kinase inhibitors in a variety of applications. However, as the number of inhibitory targets increases, side effects may also increase. Cyanidin has been consumed in natural foodstuffs for thousands of years and is regarded as safe. In this study, we demonstrated that MKK4, MEK1, and Raf-1 are molecular targets of cyanidin. Therefore, cyanidin may confer multiple health benefits by targeting these kinases.

To summarize, cyanidin inhibits UVB-induced COX-2 expression in JB6 P+ cells by blocking the MKK4, MEK1, and Raf-1 pathways (Fig. 5E). These results provide insight into the molecular action of cyanidin and indicate the potential of cyanidin as a novel chemopreventive agent.

Conflict of interest statement

None declared.

Acknowledgements

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