

Anti-inflammatory Action of Pterostilbene Is Mediated through the p38 Mitogen-Activated Protein Kinase Pathway in Colon Cancer Cells

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Abstract

Oxidative/nitrosative stress and generation of proinflammatory cytokines are hallmarks of inflammation. Because chronic inflammation is implicated in several pathologic conditions in humans, including cancers of the colon, anti-inflammatory compounds may be useful chemopreventive agents against colon cancer. Stilbenes, such as resveratrol, have diverse pharmacologic activities, which include anti-inflammation, cancer prevention, a cholesterol-lowering effect, enhanced insulin sensitivity, and increased life span. We previously showed that pterostilbene (*trans*-3,5-dimethoxy-4'-hydroxystilbene), a structural analogue of resveratrol, is present in blueberries and that pterostilbene inhibited expression of certain inflammation-related genes in the colon and suppressed aberrant crypt foci formation in rats. Here, we examined molecular mechanisms of the action of pterostilbene in colon cancer. Pterostilbene reduced cell proliferation, down-regulated the expression of c-Myc and cyclin D1, and increased the level of cleaved poly(ADP-ribose) polymerase. A combination of cytokines (tumor necrosis factor- α , IFN- γ , and bacterial endotoxin lipopolysaccharide) induced inflammation-related genes such as *inducible nitric oxide synthase* and *cyclooxygenase-2*, which was significantly suppressed by treatment with pterostilbene. We further identified upstream signaling pathways contributing to the anti-inflammatory activity of pterostilbene by investigating multiple signaling pathways, including nuclear factor- κ B, Janus-activated kinase-signal transducer and activator of transcription, extracellular signal-regulated kinase, p38, c-Jun NH₂-terminal kinase, and phosphatidylinositol 3-kinase. Cytokine induction of the p38-activating transcription factor 2 pathway was markedly inhibited by pterostilbene among the different mediators of signaling evaluated. By silencing the expression of the p38 α isoform, there was significant reduction in cytokine induction of inducible nitric oxide synthase and cyclooxygenase-2. Our data suggest that the p38 mitogen-activated protein kinase cascade is a key signal transduction pathway for eliciting the anti-inflammatory action of pterostilbene in cultured HT-29 colon cancer cells.

The intriguing link between chronic inflammation and cancer has been the subject of numerous studies for more than a century (1). In particular, the development of colon cancer is a characteristic scenario in which inflammatory conditions such as ulcerative colitis increase the risk of colon cancer by 20-fold (2). The presence of certain inflammation markers, such as the

C-reactive protein circulating in the blood, is correlated with an increased risk of colon cancer (3). In addition, overexpression of proinflammatory enzymes, such as inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), has been reported in human colon cancer (4, 5) and in an azoxymethane-induced colon cancer model in rats (6, 7). More importantly, selective inhibitors of these inflammatory genes are effective in reducing the number of colorectal polyps in humans and in suppressing the formation of azoxymethane-induced hyperplastic aberrant crypt foci and colon tumors in rats (7–10).

Epithelial cells express iNOS and COX-2 in response to inflammatory cytokines and the bacterial endotoxin lipopolysaccharide (LPS), and the transcriptional regulation of iNOS and COX-2 is complex (11–14). This process involves several transcription factors, including nuclear factor- κ B (NF- κ B), activator protein-1, CCAAT/enhancer binding protein, activating transcription factor (ATF)/cyclic AMP-responsive element binding protein, and Janus-activated kinase-signal transducer and activator of transcription (JAK-STAT) family (12, 14, 15). Depending on the cell type, various downstream

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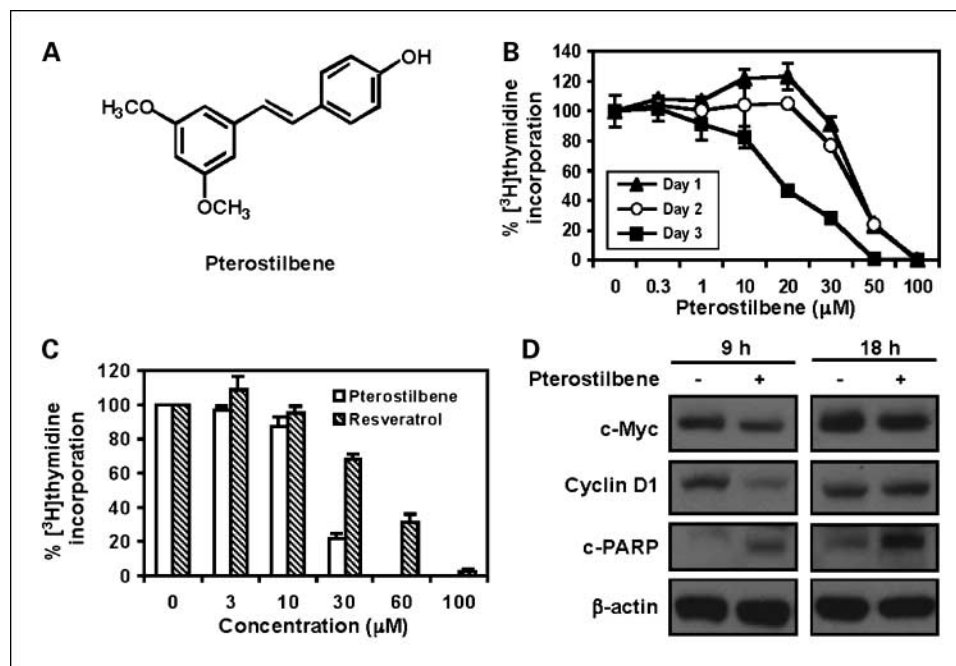


Fig. 1. Pterostilbene inhibits [^3H]thymidine incorporation into the DNA of HT-29 cells and modulates the level of proteins involved in cellular proliferation and apoptosis. **A**, structure of pterostilbene. **B**, HT-29 cells were seeded on a 24-well plate (20,000 per well) in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. The cells were incubated with pterostilbene at different concentrations or with DMSO as the control vehicle for 1, 2, or 3 d. Tritium-labeled thymidine (1 μCi ; [^3H]thymidine) per well was added and the amount of incorporated radioactivity in DNA was measured using a liquid scintillation spectrometer. The experiment was repeated twice, with each experiment done in duplicates. *Points*, mean; *bars*, SD. **C**, experiments were set up and conducted similar to the conditions as mentioned for **B**, with the exception that HT-29 cells were treated with pterostilbene, resveratrol, or DMSO as control for 3 d before [^3H]thymidine incorporation. The experiment was repeated twice, with each experiment done in duplicates. *Columns*, mean; *bars*, SD. **D**, HT-29 cells ($1.5 \times 10^6/100\text{-mm}$ dish) were treated with pterostilbene at a concentration of 50 $\mu\text{mol/L}$. The cells were harvested after 9 or 18 h of incubation with pterostilbene, and protein samples were analyzed by Western blotting.

signaling pathways are also involved in the transcriptional regulation of iNOS and COX-2.

There are several upstream kinase pathways responsible for transcriptional regulation of COX-2 and iNOS, including mitogen-activated protein kinases (MAPK). MAPKs are composed of extracellular signal-regulated kinase (ERK), p38 kinase, and c-Jun NH₂-terminal kinase (JNK; ref. 16). These MAPKs are activated by MAPK kinase (MAPKK), and once activated, these MAPKs in turn activate several transcription factors, such as Elk1, ATF2, and c-Jun, which are the major activators of iNOS and COX-2 genes (17, 18). It was reported that COX-2 expression induced by interleukin-1 β (IL-1 β) in HT-29 cells was up-regulated by all three MAPKs (19). Furthermore, p38 MAPK is the major signaling pathway, other than NF- κ B, involved in the regulation of inflammatory cytokine synthesis (20).

Recently, we showed that pterostilbene (Fig. 1), a naturally occurring analogue of resveratrol, caused suppression of aberrant crypt foci formation in the azoxymethane-induced colon cancer model in rats, which may be due to a decreased expression of inflammatory genes, such as iNOS, in the colonic crypts and in the aberrant crypt foci (21). Stilbenes, including resveratrol and pterostilbene, are present in small berries such as blueberries and deerberries (22, 23). The discovery of resveratrol as a cancer-preventive agent (24) has fostered interest in testing the cancer-preventive activity of other naturally occurring stilbenes in many laboratories (25–28). It has been reported that the bioavailability of resveratrol is low when given orally, probably

due to three hydroxyl groups (29, 30). Pterostilbene, a stilbene found in several types of blueberries, such as rabbiteye blueberries, and also in unripe Pinot noir grapes (22), has two methoxy groups and one hydroxyl group (Fig. 1), and this structural difference from resveratrol may contribute to the better bioavailability of pterostilbene *in vivo* when compared with resveratrol (29–31). Pterostilbene and resveratrol both have strong antioxidant and hypolipidemic activities (25, 32–34). However, Rimando et al. (33) have shown that pterostilbene, but not resveratrol, piceatannol, or resveratrol trimethyl ether, is a peroxisome proliferator-activated receptor α agonist, suggesting a difference in their mechanisms of action.

In the present study, we indicate that pterostilbene is more potent than resveratrol as an inhibitor of the proliferation of cultured HT-29 colon cancer cells. Although there have been some detailed studies on the chemopreventive effect of resveratrol, very little is known about the mechanism of action of pterostilbene. The present study aims to understand the inhibitory effects of pterostilbene on the induction of inflammatory markers in the HT-29 colon cancer cell line. Based on our studies, the anti-inflammatory property of pterostilbene may be regarded as a key attribute for its role against colon tumorigenesis. The effects of pterostilbene on the activation of upstream signaling pathways and transcription factors involved in NF- κ B, JAK-STAT, and MAPK pathways were investigated. Among them, p38 MAPK was identified as a key mediator for the inhibitory effect of pterostilbene on the formation of iNOS and COX-2.

Materials and Methods

Reagents

Pterostilbene (*trans*-3,5-dimethoxy-4'-hydroxystilbene; Fig. 1) and resveratrol were synthesized at the Natural Products Utilization Research Unit, United States Department of Agriculture (purity >99.9%). The compounds were dissolved in DMSO, and the final concentration of DMSO in the cell culture studies was 0.1% or less. The controls were used DMSO alone in all experiments. Recombinant human IFN- γ and tumor necrosis factor- α (TNF- α) were purchased from R&D Systems, Inc., and LPS (from *Escherichia coli* 0111:B4 γ -irradiated) was purchased from Sigma.

Cell culture

Human colon carcinoma cell line HT-29 was obtained from the American Type Culture Collection. The cells were maintained in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37°C and 5% CO₂. The cytokine mixture, consisting of 10 ng/mL TNF- α , IFN- γ , and LPS, was used to induce iNOS and COX-2, unless otherwise mentioned. The cells were treated with the test compound either alone or in combination with cytokines for different time intervals.

Measurement of cell proliferation by [³H]thymidine incorporation

HT-29 cells were plated at a density of 20,000 per well in a 24-well plate and treated with varying concentrations of pterostilbene for a period of 1, 2, or 3 d at 37°C. Before harvest, the cells

were incubated with 1 μ Ci [³H]thymidine for 4 h at 37°C and washed with PBS. The cells were precipitated with cold 10% trichloroacetic acid for 10 min and solubilized with 0.5 mL solubilization buffer (0.2 mol/L NaOH, 40 μ g/mL salmon sperm DNA) for 2 h at room temperature. The lysate was transferred to 5 mL Ecolume, and the [³H]thymidine incorporated into the DNA of HT-29 cells was determined using a scintillation spectrometer (Beckman Coulter).

Western blot analysis

Whole-cell and nuclear protein extracts from different experiments were collected and analyzed by Western blotting. The protein samples were separated on 4% to 15% SDS-PAGE gels (Bio-Rad) followed by transfer to a polyvinylidene difluoride membrane. The membranes were blocked with 5% milk in Tris buffer for 1 h and then incubated with the appropriate primary antibody solutions overnight at 4°C. The membranes were washed with Tris buffer and incubated with horseradish peroxidase-conjugated secondary antibody solutions for 1 h at room temperature. The protein bands were visualized using a chemiluminescence-based kit from Amersham Biosciences. The primary antibodies against iNOS, COX-2, I κ B α , p65, cyclin D1, c-Myc (Santa Cruz Biotechnology), phospho-STAT3 (p-STAT3), phospho-STAT1 (p-STAT1), phospho-ERK1/2 (p-ERK1/2), phospho-JNK1/2 (p-JNK1/2), phospho-p38 (p-p38), phospho-Akt (p-Akt), phospho-ATF2 (p-ATF2), phospho-Elk1 (p-Elk1), poly(ADP-ribose) polymerase (PARP), p38 α , p38 β , total p38 (Cell Signaling Technology, Inc.), and actin (Sigma) and secondary antibodies (Santa Cruz Biotechnology) were used.

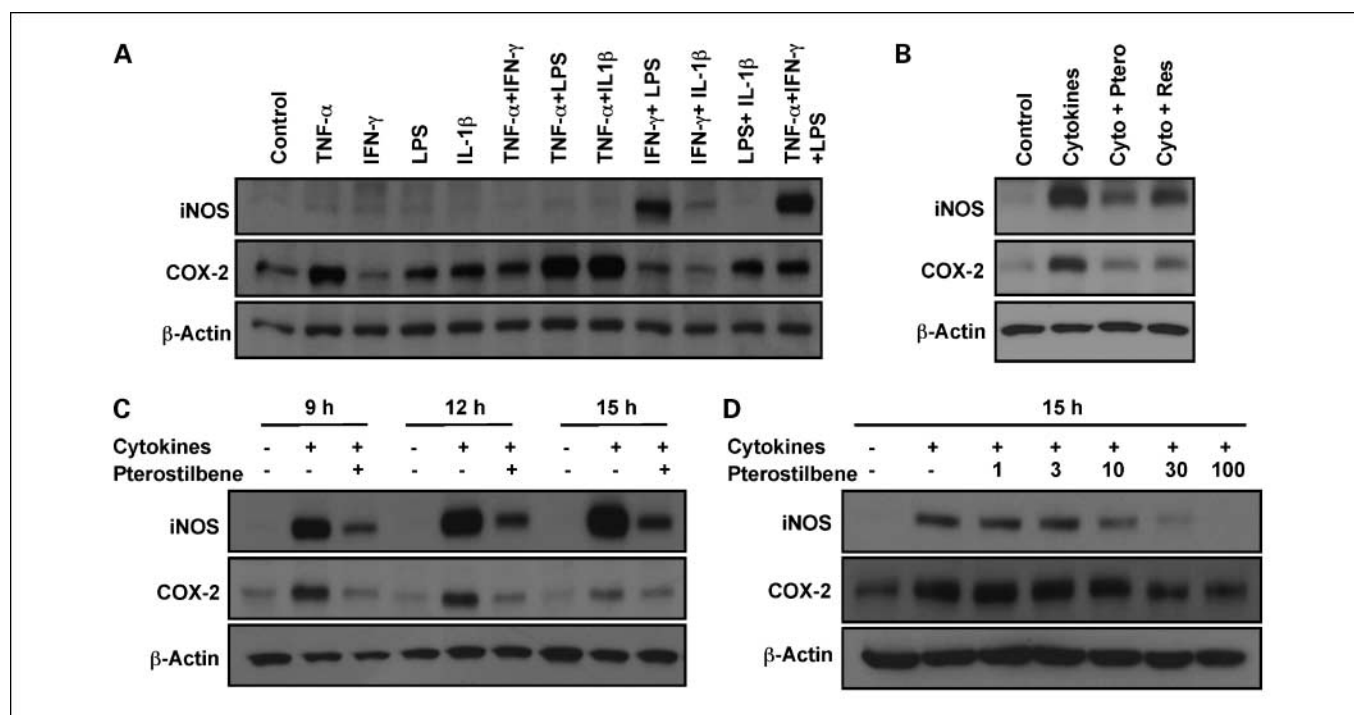


Fig. 2. Effect of cytokines and pterostilbene on the level of iNOS and COX-2 protein in HT-29 cells. **A**, iNOS and COX-2 proteins are induced differentially by various cytokines. HT-29 cells (1.5×10^6 /100-mm dish) were treated with TNF- α , IFN- γ , LPS, and IL-1 β (each at 10 ng/mL) either alone or in different combinations. The cells were harvested for protein measurements after 15 h, and the samples were immunoblotted to determine induction of iNOS and COX-2. β -Actin levels were used as the loading control. **B**, comparison of pterostilbene and resveratrol. HT-29 cells (1.5×10^6 /100-mm dish) were treated with a mixture of TNF- α , IFN- γ , and LPS (each at 10 ng/mL; labeled as *Cytokines* or *Cyto*) with or without pterostilbene (*Ptero*; 30 μ M/L) or resveratrol (*Res*; 30 μ M/L) for 15 h. The protein levels of iNOS and COX-2 were determined. **C**, iNOS and COX-2 induction by the cytokine mixture is time dependent: effect of pterostilbene. HT-29 cells (1.5×10^6 /100-mm dish) were treated with the combination of TNF- α , IFN- γ , and LPS, each at 10 ng/mL, for 9, 12, or 15 h in the presence or absence of pterostilbene (50 μ M/L). The protein levels of iNOS and COX-2 were determined. **D**, pterostilbene blocks the induction of iNOS and COX-2 in a dose-dependent manner. HT-29 cells (1.5×10^6 /100-mm dish) were treated with a mixture of TNF- α , IFN- γ , and LPS (each at 10 ng/mL) in the presence or absence of different concentrations of pterostilbene for 15 h. The protein levels of iNOS and COX-2 were determined by Western blot analysis.

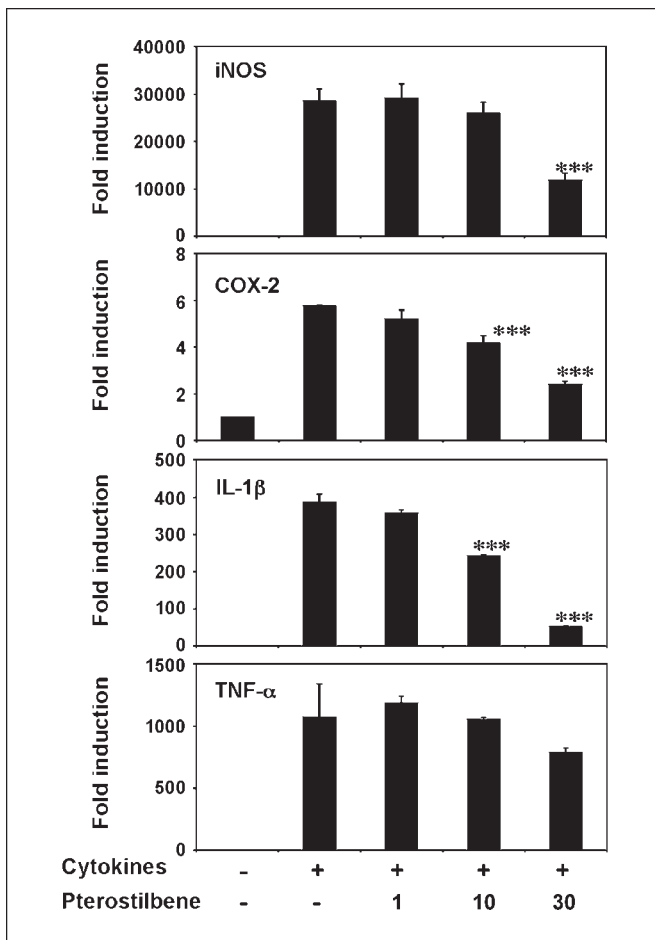


Fig. 3. Cytokine-induced formation of mRNA for iNOS, COX-2, IL-1 β , and TNF- α : effect of pterostilbene. HT-29 cells (1.5×10^6 /100-mm dish) were treated with a mixture of TNF- α , IFN- γ , and LPS (each at 10 ng/mL) alone or together with different concentrations of pterostilbene (μ mol/L) for 9 h. Total RNA was isolated and mRNA levels for iNOS, COX-2, TNF- α , and IL-1 β were measured using quantitative RT-PCR analysis as described in Materials and Methods. Columns, mean; bars, SD. Statistical significance was calculated by the Student's *t* test ($n = 4$; ***, $P < 0.001$).

Quantitative reverse transcription-PCR analysis

The procedure for quantitative reverse transcription-PCR (RT-PCR) analysis is previously reported (35). Briefly, the cells were incubated with compounds for indicated period and the cells were then lysed using Trizol to extract RNA. RNA was reverse transcribed into cDNA using a high-capacity cDNA archive kit (Applied Biosystems). The cDNA was used for quantitative PCR, which was run on the ABI Prism 700 Sequence Detection System. The primers for the iNOS, COX-2, IFN- γ , TNF- α , IL-1 β , and glyceraldehyde-3-phosphate dehydrogenase were obtained from Applied Biosystems.

Fluorescence microscopy

HT-29 cells were incubated in a chamber slide (Nunc) with cytokines and pterostilbene for 15 and 30 min to detect p-p38 and p-ATF2, respectively. Cells were fixed with 4% paraformaldehyde [1 \times PBS (pH 7.4)] for 20 min and blocked with 10% bovine serum albumin/0.5% Triton X-100/1 \times PBS for 1 h. Following this, the cells were incubated sequentially with primary antibody (1:100 dilution for p-p38 or 1:25 dilution for p-ATF2) overnight and fluorophore-conjugated secondary antibody (Alexa Fluor 488, Invitrogen) for 1 h and 4',6-diamidino-2-phenylindole (DAPI) for 30 min. The cells were irra-

diated with green laser at 488 nm for detection of p-p38 and p-ATF2 and UV light at 364 nm for nuclear staining by DAPI.

Small interfering RNA transfection study

Small interfering RNA (siRNA) against p38 α and p38 β isoforms was purchased from Dharmacon. HT-29 cells were transfected as given in the manufacturer's protocol. Briefly, the cells were plated at 150,000 per well in six-well plates. Individual siRNA was mixed with Accell siRNA delivery medium and added to the wells to give a final concentration of 1 μ mol/L per well. After incubation with the mixture for 72 h, the medium was changed to complete medium and cells were treated with cytokine mixture and pterostilbene for an additional 15 h. Changes in induction of iNOS and COX-2 were measured by Western blot analysis.

Statistical analysis

Quantitative data are reported as the mean \pm SD for the individual experiments as specified in the figure legends. Statistical significance analysis was done using the Student's *t* test. The number of observations for each treatment, represented as *n*, and the measure of significance of treatments, the *P* value, are given in the figure legends.

Results

Pterostilbene inhibits the proliferation of cultured colon cancer HT-29 cells

We evaluated the effect of pterostilbene and resveratrol on the growth of cultured colon cancer HT-29 cells. The cells were incubated with different concentrations of pterostilbene for 1, 2, and 3 days, and cell proliferation was estimated by measuring [3 H]thymidine incorporated into DNA. The 3-day incubation gave the strongest growth inhibition, and there was a dose-dependent effect (Fig. 1B). As illustrated in Fig. 1C, pterostilbene was a more potent inhibitor of proliferation (IC₅₀, 22.4 μ mol/L) when compared with resveratrol treatment (IC₅₀, 43.8 μ mol/L) under the same conditions. IC₅₀ values were determined using TableCurve 2D software (version 5.01) from Systat. To evaluate whether pterostilbene potentiates cell cycle arrest or apoptosis in HT-29 cells, we examined the effect of pterostilbene on proteins regulating the cell cycle/apoptosis pathways. Pterostilbene was effective in reducing c-Myc and cyclin D1 levels after a 9-hour incubation (Fig. 1C). However, pterostilbene showed no induction of p21 and p27, which belong to the CIP-KIP family of cyclin-dependent kinase inhibitors (data not shown). As a marker for the induction of apoptosis, we determined the level of cleaved PARP. Treatment with pterostilbene for 9 or 18 hours increased the level of cleaved PARP (Fig. 1D).

Cytokines act synergistically to induce iNOS and COX-2 in HT-29 cells

To determine the cytokines or the combination of cytokines that will give maximal induction of iNOS and COX-2 in HT-29 cells, we treated the cells with TNF- α , IFN- γ , LPS, and IL-1 β either alone or in combination for 15 hours (Fig. 2A). Addition of the cytokines individually to HT-29 cells did not cause a noticeable induction of iNOS. Although the addition of IFN- γ plus LPS caused a strong induction, even stronger induction of iNOS among the combinations tested was exhibited by a triple combination of TNF- α , IFN- γ , and LPS. COX-2 was induced by TNF- α , LPS, or IL-1 β individually, and TNF- α was the most potent inducer. The combination of TNF- α with either IL-1 β or LPS yielded the strongest induction of COX-2.

The triple combination induced both iNOS and COX-2, and this combination was selected for additional studies. Because pterostilbene is a naturally occurring analogue of resveratrol, we first compared the inhibitory effects of pterostilbene and resveratrol against the induction of iNOS and COX-2 protein in this condition. At the concentration tested (30 $\mu\text{mol/L}$), pterostilbene showed better inhibitory activity than resveratrol against induction of iNOS and COX-2 proteins (Fig. 2B).

Cytokine induction of iNOS and COX-2 is time dependent, and pterostilbene dose dependently blocks the induction

To determine the kinetics of induction of iNOS and COX-2 in HT-29 colon cancer cells, the cells were treated with the cytokine mixture of TNF- α , IFN- γ , and LPS for periods of 9, 12, and 15 hours. The induction of iNOS was highest at 15 hours, whereas the COX-2 level was high at 9 to 12 hours and low at 15 hours (Fig. 2C). These data show that maximal induction of COX-2 occurs earlier than that of iNOS. As also shown in Fig. 2C, pterostilbene at 50 $\mu\text{mol/L}$ markedly blocked the induction of iNOS and COX-2 by the cytokine mixture at each time point. In addition, we determined the effect of treatment of the cells with different concentrations of pterostilbene on the induction of iNOS and COX-2 by the cytokine mixture. Pterostilbene inhibited the induction of iNOS and COX-2 in a dose-dependent manner (Fig. 2D).

Pterostilbene down-regulates mRNA levels of inflammatory genes iNOS and COX-2 and proinflammatory cytokines IL-1 β and TNF- α

The gene-mediated expression of iNOS and COX-2 is regulated both at the transcriptional and translational levels (13, 14). To evaluate the effect of pterostilbene on cytokine-induced expression of proinflammatory enzymes and cytokines, the induction of mRNA levels of *iNOS*, *COX-2*, *IL-1 β* , *IFN- γ* , and *TNF- α* genes was analyzed by quantitative RT-PCR after HT-29 cells were treated with cytokines and/or pterostilbene. Pterostilbene at 30 $\mu\text{mol/L}$ strongly inhibited iNOS, COX-2, and IL-1 β mRNA induction by the cytokine mixture (Fig. 3). Induction of TNF- α mRNA by cytokines was observed, but pterostilbene showed only a weak inhibitory effect (Fig. 3). We also measured the mRNA level of IFN- γ induced by the cytokine mixtures, but it was too low to be detected in HT-29 cells (data not shown).

Pterostilbene blocks the activation of p38 signaling in colon cancer cells

Cytokines induce iNOS and COX-2 through various signaling pathways (13, 15). To elucidate the mechanism responsible for the anti-inflammatory action of pterostilbene, we examined the upstream pathways for iNOS and COX-2 formation, which are activated rapidly after cytokine treatment. As shown in Fig. 4A, the NF- κB /I $\kappa\text{B}\alpha$ and JAK-STAT pathways were investigated. Cytokine treatment for a short time (15 minutes) decreased I $\kappa\text{B}\alpha$ levels. Pterostilbene, however, did not block the degradation of I $\kappa\text{B}\alpha$ protein induced by the cytokines (Fig. 4A). In addition, the accumulation of the p65 subunit of NF- κB in the nucleus was not affected by pterostilbene (data not shown), confirming that NF- κB signaling is not regulated by pterostilbene in colon cancer cells.

The significance of the JAK-STAT pathway in HT-29 cells was evaluated by the level of p-STAT1 and p-STAT3 proteins. Cytokines activated the STAT pathway, as shown by a strong induction of p-STAT1 and p-STAT3. However, pterostilbene did not alter the level of induced p-STAT1 and p-STAT3 (Fig. 4A). When we determined the activation of ERK1/2 and p38 kinases by cytokines by measuring the levels of phosphorylated ERK1/2 and p38, we found that pterostilbene did not block ERK1/2 activation but strongly inhibited activation of p38 (Fig. 4B). Cytokine-induced increase in p-JNK protein was noticeable, but there was little or no inhibitory effect of pterostilbene on this increase. Cytokine or pterostilbene treatment did not change the level of p-Akt, which is the downstream effector of the phosphatidylinositol 3-kinase pathway (Fig. 4B).

The p38-ATF2 MAPK pathway is inhibited by pterostilbene in colon cancer cells

Because pterostilbene is effective in down-regulating the cytokine-induced activation of p38, we further examined the involvement of pterostilbene on some of the known upstream effectors and downstream targets of p38 kinase. Phospho-MKK3/6 is known as the major molecule responsible for activating p38 MAPK, which, in turn, is activated by the upstream kinase in the MAPK cascade. We found that pterostilbene was effective in inhibiting cytokine-induced phosphorylation of MKK3/6 at 15 minutes (Fig. 5A). Furthermore, the activation (phosphorylation) of well-known downstream targets of p38, ATF2 and Elk1, was also blocked by pterostilbene at 30 minutes (Fig. 5B).

Changes in the intracellular expression pattern of p-p38 and p-ATF2 were detected by immunofluorescence. With regard to p-p38, cytokine treatment induced p-p38 and its localization mainly in the nucleus and perinuclear region. Recently, Siddiqui et al. (36) have reported similar localization pattern for activated p38 in endothelial cells. Pterostilbene treatment attenuated this increase (Fig. 5C), parallel to the observations from Western blot analysis (Fig. 4B). Activated transcription

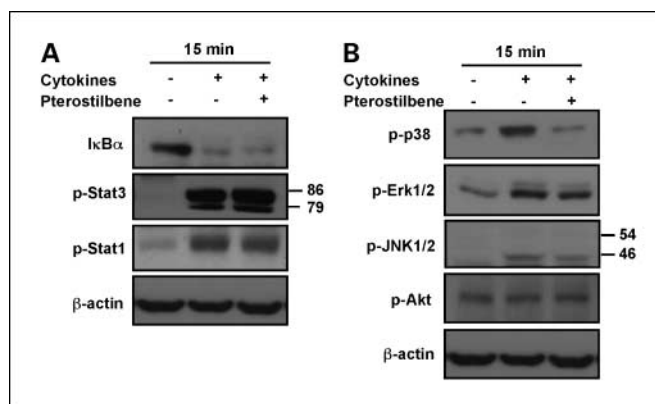


Fig. 4. Evaluation of different cellular signaling pathways affected by pterostilbene in HT-29 cells. *A*, pterostilbene showed little or no effects on the NF- κB and JAK-STAT pathways. *B*, pterostilbene effectively blocks activation of p38 MAPK signaling. HT-29 cells ($1.5 \times 10^6/100\text{-mm}$ dish) were incubated with a mixture of TNF- α , IFN- γ , and LPS (each at 10 ng/mL) and pterostilbene (30 $\mu\text{mol/L}$) for 15 min. The cells were harvested and protein samples were immunoblotted for I $\kappa\text{B}\alpha$, p-STAT3, p-STAT1, p-ERK1/2, p-p38, p-JNK1/2, p-Akt, and β -actin.

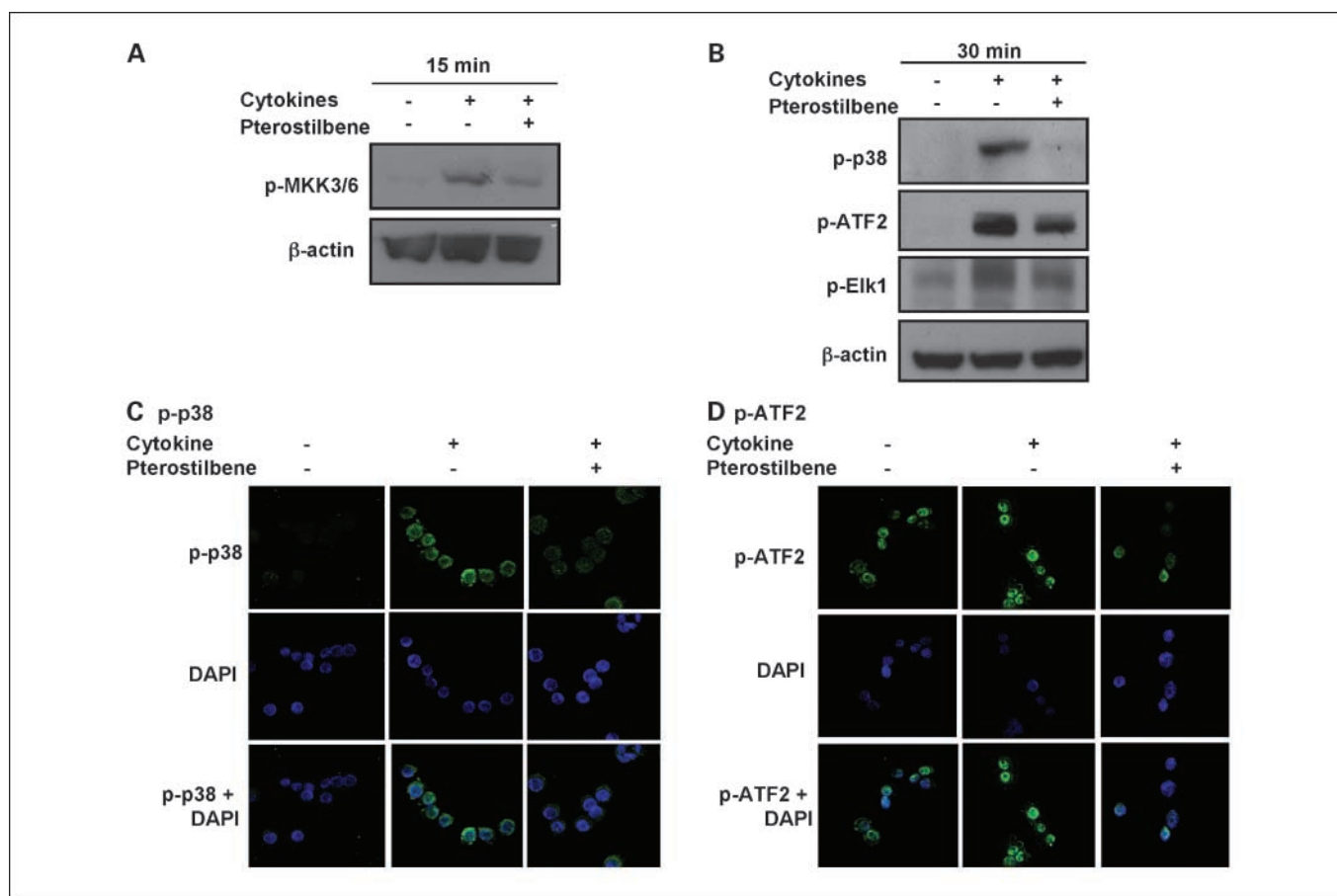


Fig. 5. Effect of pterostilbene on the p38-ATF2 pathway. **A**, pterostilbene down-regulates the phosphorylation of upstream kinase of p38, MKK3/6. HT-29 cells (1.5×10^6 /100-mm dish) were incubated with a mixture of TNF- α , IFN- γ , and LPS (each at 10 ng/mL) in the presence or absence of pterostilbene (30 μ mol/L) for 15 min, and protein samples were immunoblotted for p-MKK3/6 and β -actin. **B**, pterostilbene down-regulates the phosphorylation of downstream targets of p38. HT-29 cells (1.5×10^6 /100-mm dish) were incubated with a mixture of TNF- α , IFN- γ , and LPS (each at 10 ng/mL) in the presence or absence of pterostilbene (30 μ mol/L) for 30 min, and protein samples were immunoblotted for p-p38, p-Elk1, p-ATF2, and β -actin. **C**, pterostilbene abolishes nuclear accumulation of p-p38 induced by cytokine mixture. HT-29 cells (30,000 per chamber in a 4-well chamber slide) were incubated with a mixture of TNF- α , IFN- γ , and LPS (each at 10 ng/mL) and pterostilbene (30 μ mol/L) for 15 min. Green, staining for p-p38; blue, nuclear staining by DAPI. Magnification, $\times 63$. **D**, pterostilbene abolishes nuclear accumulation of p-ATF2 induced by cytokine mixture. HT-29 cells (30,000 per chamber in a 4-well chamber slide) were incubated with a mixture of TNF- α , IFN- γ , and LPS (each at 10 ng/mL) and pterostilbene (30 μ mol/L) for 30 min. Green, staining for p-ATF2; blue, nuclear staining by DAPI. Magnification, $\times 63$.

factor, p-ATF2, was prominent in the nucleus by cytokine treatment and pterostilbene virtually nullified these elevated levels (Fig. 5D). Thus, significantly lower levels of the activated p38 and ATF2 were observed in the nucleus of HT-29 cells after treatment with the stilbene.

p38 α isoform is crucial for the induction of iNOS and COX-2 in HT-29 colon cancer cells by the cytokine mixture

Four mammalian p38 isoforms, p38 α , p38 β , p38 γ , and p38 δ , have been identified: p38 α and p38 β forms are ubiquitously expressed, whereas p38 γ is present mostly in skeletal muscle, heart, lung, and thymus, and p38 δ in lungs, pancreas, testis, kidney, and small intestine (37). SB203580, a pharmacologic inhibitor that specifically targets the p38 α and p38 β isoforms (38), was shown to lower cytokine induction of iNOS and COX-2 in our study (data not shown). Thus, to delineate the role of different isoforms of p38 MAPK for the induction of iNOS and COX-2, we used siRNA against p38 α and p38 β . Results showed that absence of p38 α expression almost com-

pletely blocked the induction of iNOS (Fig. 6). Because deletion of p38 α expression by itself resulted in almost no induction of iNOS, there was hardly any change by cotreatment of pterostilbene with the cytokine mixture in the p38 α siRNA-treated group. In addition, siRNA against p38 α also markedly reduced COX-2 induction. Moreover, p38 α is the most abundant isoform of p38 (Fig. 6, total p38 blot). These results suggest that p38 α is the key molecule in inducing iNOS and COX-2 with the cytokine mixture, and pterostilbene may be acting through this p38 α isoform to block the inflammatory enzyme expression in HT-29 cells.

Discussion

In the present study, we investigated the mechanisms of action of pterostilbene in HT-29 colon cancer cells. The results of our study indicate that pterostilbene is more effective than resveratrol as an inhibitor of DNA synthesis in the human adenocarcinoma HT-29 cell line (Fig. 1C). In addition, pterostilbene showed better activity than resveratrol for inhibiting the induction of inflammatory genes, such

as iNOS and COX-2 (Fig. 2B). The better activity of pterostilbene over resveratrol may, in part, be explained by structural differences. Pterostilbene with two methoxy groups and one hydroxyl group has improved lipophilicity and a better potential for cellular uptake compared with resveratrol, which has trihydroxy groups. We showed that pterostilbene inhibited the growth of HT-29 cells and altered markers of cellular proliferation and apoptosis, as shown by lower protein levels of c-Myc and cyclin D1 as well as an increased level of cleaved PARP in pterostilbene-treated cells (Fig. 1). These data are consistent with the results of recent studies indicating that pterostilbene and resveratrol induce apoptosis and down-regulate genes that are directly involved in cell proliferation, including *cyclin D1*, *in vivo* and *in vitro* (39, 40).

In our study with HT-29 colon cancer cells, a triple combination of TNF- α , IFN- γ , and LPS resulted in a marked induction of iNOS and COX-2 (Fig. 2A), and pterostilbene reduced the induction of iNOS and COX-2 in a dose-dependent fashion (Fig. 2D). Quantitative RT-PCR data showed that the regulation of iNOS and COX-2 occurred at the transcriptional level with pterostilbene effectively down-regulating the cytokine induction of iNOS and COX-2 mRNA (Fig. 3). Treatment with a mixture of cytokines induced mRNA synthesis for proinflammatory cytokines, such as IL-1 β , and this was significantly inhibited by pterostilbene. These results underscore the anti-inflammatory potential of pterostilbene.

TNF- α , IFN- γ , IL-1 β , and LPS are effective inducers of the expression of inflammatory genes in macrophages and epithelial cells, although expression levels vary with cell type (41). The up-regulation of iNOS and COX-2 is mediated by multiple pathways, which vary with cell type and cytokines used. The involvement of NF- κ B, activator protein-1, MAPKs, and JAK-STAT in the expression of these genes has been evaluated for a variety of compounds with anti-inflammatory potential. Resveratrol, which is structurally similar to pterostilbene, reduced iNOS and COX-2 induction in rat glioma cells and inhibited iNOS induction by LPS in macrophages by reducing NF- κ B (42, 43). Recently, pterostilbene was found to suppress the activation of ERK, p38, phosphatidylinositol 3-kinase, and NF- κ B in LPS-induced murine macrophages, suggesting that these pathways play crucial roles in the action of pterostilbene to inhibit iNOS and COX-2 in macrophages (44). However, our results show the p38 MAPK cascade as a major signaling pathway inhibited by pterostilbene in HT-29 colon cancer cells, suggesting that cell type specificity may contribute to this difference (Fig. 4). In addition, our preliminary data revealed that pterostilbene does not affect cell proliferation-related events via the p38 pathway (data not shown), indicating that there may be two distinct mechanisms of pterostilbene for its antiproliferation and anti-inflammatory actions.

The p38 MAPK cascade is activated by its upstream kinase MKK3/6, which is the MAPKK for p38, and we found that pterostilbene strongly inhibited the activation of both MKK3/6 and p38 (Fig. 5A and B). This suggests that pterostilbene may activate p38 MAPK through the conventional kinase cascade that has small GTP proteins, such as Rac, Rho, cdc42 acting on MAPKKK or MAP3K followed by its substrate, MAPKK or MAP2K (MKK3/6), and finally p38 MAPK (45). In the MAPK cascade, we also observed that pterostilbene acts on downstream targets of p38, namely, ATF2 and

Elk1 (Fig. 5B). There are a myriad of transcription factors and kinases that are affected by p38, such as MEF2, MSK, CHOP, and MAPKAP (46), but we examined two key mediators, ATF2 and Elk1, which are known to play important roles in inflammatory gene responses (47). ATF2 is a subunit of the activator protein-1 complex and binds to the CRE promoter sequence on iNOS and COX-2, and Elk1 belongs to the ETS transcription factor and binds to the ETS DNA-binding domain on the promoter sequence of inflammatory genes (14). In the present study, we found that pterostilbene blocked the phosphorylation and nuclear translocation of p38 and ATF2 induced by cytokine mixture (Fig. 5B-D), providing evidence for the involvement of the p38 MAPK-ATF2 pathway for the anti-inflammatory action of pterostilbene in HT-29 colon cancer cells.

Among the different isoforms of p38 MAPKs, p38 α is known to play a key role in inflammatory processes (48). This MAPK was originally identified as a molecular target of the pyridinyl imidazole class of compounds, such as SB203580, which were known to inhibit proinflammatory cytokine synthesis, and many of these inhibitors have entered clinical trials for inflammatory diseases (38). Because the p38 inhibitor SB203580 suppresses activation of both p38 α and p38 β , it is difficult to distinguish the effects produced by each isoform independently. In our study, we used RNA interference to show that p38 α is the most abundant isoform in HT-29 colon cancer cells, and p38 α is the key molecule involved in iNOS and COX-2 expression (Fig. 6). This observation along with coordinated results from short time point experiments (Figs. 4 and 5) indicate that pterostilbene may inhibit iNOS and COX-2 expression primarily through its action on p38 α MAPK.

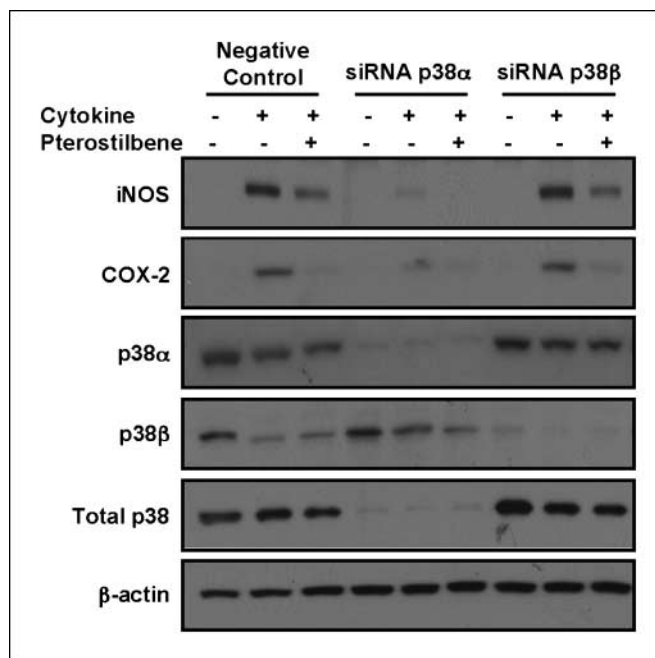


Fig. 6. Effect of pterostilbene on the induction of iNOS and COX-2 on knocking down p38 α and p38 β . HT-29 cells (150,000 per well in six-well plate) were transfected with siRNA against p38 α and p38 β and incubated in serum-free Accell delivery medium for 72 h. Cells were then incubated with a mixture of TNF- α , IFN- γ , and LPS (each at 10 ng/mL) in the presence or absence of pterostilbene (30 μ mol/L) for an additional 15 h. Cells were harvested for protein and the samples were analyzed by Western blotting.

In conclusion, the data presented here indicate that pterostilbene inhibits growth and exerts an anti-inflammatory action in HT-29 colon cancer cells. Pterostilbene-induced inhibition of p38 MAPK signaling may be a key effect of pterostilbene required for reduction of inflammatory markers, such as iNOS and COX-2. Overall, pterostilbene is a promising anti-inflammatory agent for inhibition of colon carcinogenesis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

- Schafer M, Werner S. Cancer as an overheating wound: an old hypothesis revisited. *Nat Rev Mol Cell Biol* 2008;9:628–38.
- Ulrich S, Loitsch SM, Rau O, et al. Peroxisome proliferator-activated receptor γ as a molecular target of resveratrol-induced modulation of polyamine metabolism. *Cancer Res* 2006;66:7348–54.
- Erlinger TP, Platz EA, Rifai N, Helzlsouer KJ. C-reactive protein and the risk of incident colorectal cancer. *JAMA* 2004;291:585–90.
- Cianchi F, Cortesini C, Fantappie O, et al. Inducible nitric oxide synthase expression in human colorectal cancer: correlation with tumor angiogenesis. *Am J Pathol* 2003;162:793–801.
- Ohta T, Takahashi M, Ochiai A. Increased protein expression of both inducible nitric oxide synthase and cyclooxygenase-2 in human colon cancers. *Cancer Lett* 2006;239:246–53.
- Rao CV, Kawamori T, Hamid R, Reddy BS. Chemoprevention of colonic aberrant crypt foci by an inducible nitric oxide synthase-selective inhibitor. *Carcinogenesis* 1999;20:641–4.
- Takahashi M, Mutoh M, Shoji Y, et al. Suppressive effect of an inducible nitric oxide inhibitor, ONO-1714, on AOM-induced rat colon carcinogenesis. *Nitric Oxide* 2006;14:130–6.
- Rao CV, Indranie C, Simi B, Manning PT, Connor JR, Reddy BS. Chemopreventive properties of a selective inducible nitric oxide synthase inhibitor in colon carcinogenesis, administered alone or in combination with celecoxib, a selective cyclooxygenase-2 inhibitor. *Cancer Res* 2002;62:165–70.
- Pereg D, Lishner M. Non-steroidal anti-inflammatory drugs for the prevention and treatment of cancer. *J Intern Med* 2005;258:115–23.
- Tuma R. Drugs to prevent colon cancer show promise, but hurdles remain for chemoprevention. *J Natl Cancer Inst* 2008;100:764–6.
- Kang YJ, Mbye UR, DeLong CJ, Wada M, Smith WL. Regulation of intracellular cyclooxygenase levels by gene transcription and protein degradation. *Prog Lipid Res* 2007;46:108–25.
- Chun KS, Surh YJ. Signal transduction pathways regulating cyclooxygenase-2 expression: potential molecular targets for chemoprevention. *Biochem Pharmacol* 2004;68:1089–100.
- Kleinert H, Pautz A, Linker K, Schwarz PM. Regulation of the expression of inducible nitric oxide synthase. *Eur J Pharmacol* 2004;500:255–66.
- Kracht M, Saklatvala J. Transcriptional and post-transcriptional control of gene expression in inflammation. *Cytokine* 2002;20:91–106.
- Tsatsanis C, Androulidaki A, Venihaki M, Margioris AN. Signalling networks regulating cyclooxygenase-2. *Int J Biochem Cell Biol* 2006;38:1654–61.
- Dhillon AS, Hagan S, Rath O, Kolch W. MAP kinase signalling pathways in cancer. *Oncogene* 2007;26:3279–90.
- Johnson GL, Lapadat R. Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. *Science* 2002;298:1911–2.
- Saklatvala J. The p38 MAP kinase pathway as a therapeutic target in inflammatory disease. *Curr Opin Pharmacol* 2004;4:372–7.
- Liu W, Reinmuth N, Stoeltzing O, et al. Cyclooxygenase-2 is up-regulated by interleukin-1 β in human colorectal cancer cells via multiple signaling pathways. *Cancer Res* 2003;63:3632–6.
- Lee JC, Laydon JT, McDonnell PC, et al. A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. *Nature* 1994;372:739–46.
- Suh N, Paul S, Hao X, et al. Pterostilbene, an active constituent of blueberries, suppresses aberrant crypt foci formation in the azoxymethane-induced colon carcinogenesis model in rats. *Clin Cancer Res* 2007;13:350–5.
- Rimando AM, Kalt W, Magee JB, Dewey J, Ballington JR. Resveratrol, pterostilbene, and piceatannol in *Vaccinium* berries. *J Agric Food Chem* 2004;52:4713–9.
- Roupe KA, Remsburg CM, Yáñez JA, Davies NM. Pharmacometrics of stilbenes: sequencing towards the clinic. *Curr Clin Pharmacol* 2006;1:81–101.
- Jang M, Cai L, Udeani GO, et al. Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. *Science* 1997;275:218–20.
- Rimando AM, Cuendet M, Desmarchelier C, Mehta RG, Pezzuto JM, Duke SO. Cancer chemopreventive and antioxidant activities of pterostilbene, a naturally occurring analogue of resveratrol. *J Agric Food Chem* 2002;50:3453–7.
- Aggarwal BB, Bhardwaj A, Aggarwal RS, Seeram NP, Shishodia S, Takada Y. Role of resveratrol in prevention and therapy of cancer: preclinical and clinical studies. *Anticancer Res* 2004;24:2783–840.
- Baur JA, Sinclair DA. Therapeutic potential of resveratrol: the *in vivo* evidence. *Nat Rev Drug Discov* 2006;5:493–506.
- Baur JA, Pearson KJ, Price NL, et al. Resveratrol improves health and survival of mice on a high-calorie diet. *Nature* 2006;444:337–42.
- Asensi M, Medina I, Ortega A, et al. Inhibition of cancer growth by resveratrol is related to its low bioavailability. *Free Radic Biol Med* 2002;33:387–98.
- Ferrer P, Asensi M, Segarra R, et al. Association between pterostilbene and quercetin inhibits metastatic activity of B16 melanoma. *Neoplasia* 2005;7:37–47.
- Remsburg CM, Yanez JA, Ohgami Y, Vega-Villa KR, Rimando AM, Davies NM. Pharmacometrics of pterostilbene: preclinical pharmacokinetics and metabolism, anticancer, antiinflammatory, antioxidant and analgesic activity. *Phytother Res* 2007.
- Stivala LA, Savio M, Carafoli F, et al. Specific structural determinants are responsible for the antioxidant activity and the cell cycle effects of resveratrol. *J Biol Chem* 2001;276:22586–94.
- Rimando AM, Nagmani R, Feller DR, Yokoyama W. Pterostilbene, a new agonist for the peroxisome proliferator-activated receptor α -isoform, lowers plasma lipoproteins and cholesterol in hypercholesterolemic hamsters. *J Agric Food Chem* 2005;53:3403–7.
- Miura D, Miura Y, Yagasaki K. Hypolipidemic action of dietary resveratrol, a phytoalexin in grapes and red wine, in hepatoma-bearing rats. *Life Sci* 2003;73:1393–400.
- Lee HJ, Wislocki A, Goodman C, et al. A novel vitamin D derivative activates bone morphogenetic protein signaling in MCF10 breast epithelial cells. *Mol Pharmacol* 2006;69:1840–8.
- Siddiqui SS, Siddiqui ZK, Uddin S, Minshall RD, Malik AB. p38 MAPK activation coupled to endocytosis is a determinant of endothelial monolayer integrity. *Am J Physiol Lung Cell Mol Physiol* 2007;292:L114–24.
- Ono K, Han J. The p38 signal transduction pathway: activation and function. *Cell Signal* 2000;12:1–13.
- Kumar S, Boehm J, Lee JC. p38 MAP kinases: key signalling molecules as therapeutic targets for inflammatory diseases. *Nat Rev Drug Discov* 2003;2:717–26.
- Ferrer P, Asensi M, Priego S, et al. Nitric oxide mediates natural polyphenol-induced Bcl-2 down-regulation and activation of cell death in metastatic B16 melanoma. *J Biol Chem* 2007;282:2880–90.
- Schneider Y, Duranton B, Gosse F, Schleiffer R, Seiler N, Raul F. Resveratrol inhibits intestinal tumorigenesis and modulates host-defense-related gene expression in an animal model of human familial adenomatous polyposis. *Nutr Cancer* 2001;39:102–7.
- Nathan C. Nitric oxide as a secretory product of mammalian cells. *FASEB J* 1992;6:3051–64.
- Tsai SH, Lin-Shiau SY, Lin JK. Suppression of nitric oxide synthase and the down-regulation of the activation of NF κ B in macrophages by resveratrol. *Br J Pharmacol* 1999;126:673–80.
- Kim YA, Lim SY, Rhee SH, et al. Resveratrol inhibits inducible nitric oxide synthase and cyclooxygenase-2 expression in β -amyloid-treated C6 glioma cells. *Int J Mol Med* 2006;17:1069–75.
- Pan MH, Chang YH, Tsai ML, et al. Pterostilbene suppressed lipopolysaccharide-induced up-expression of iNOS and COX-2 in murine macrophages. *J Agric Food Chem* 2008;56:7502–9.
- Ashwell JD. The many paths to p38 mitogen-activated protein kinase activation in the immune system. *Nat Rev Immunol* 2006;6:532–40.
- Kaminska B. MAPK signalling pathways as molecular targets for anti-inflammatory therapy—from molecular mechanisms to therapeutic benefits. *Biochim Biophys Acta* 2005;1754:253–62.
- Reimold AM, Kim J, Finberg R, Glimcher LH. Decreased immediate inflammatory gene induction in activating transcription factor-2 mutant mice. *Int Immunol* 2001;13:241–8.
- Hale KK, Trollinger D, Rihaneh M, Manthey CL. Differential expression and activation of p38 mitogen-activated protein kinase α , β , γ , and δ in inflammatory cell lineages. *J Immunol* 1999;162:4246–52.