Substituted *trans*-Stilbenes, Including Analogues of the Natural Product Resveratrol, Inhibit the Human Tumor Necrosis Factor Alpha-Induced Activation of Transcription Factor Nuclear Factor KappaB

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The transcription factor nuclear factor kappaB (NF-κB), which regulates expression of numerous antiinflammatory genes as well as genes that promote development of the prosurvival, antiapoptotic state is up-regulated in many cancer cells. The natural product resveratrol, a polyphenolic *trans*-stilbene, has numerous biological activities and is a known inhibitor of activation of NF-κB, which may account for some of its biological activities. Resveratrol exhibits activity against a wide variety of cancer cells and has demonstrated activity as a cancer chemopreventive against all stages, i.e., initiation, promotion, and progression. The biological activities of resveratrol are often ascribed to its antioxidant activity. Both antioxidant activity and biological activities of analogues of resveratrol depend upon the number and location of the hydroxy groups. In the present study, phenolic analogues of resveratrol and a series of substituted *trans*-stilbenes without hydroxy groups were compared with resveratrol for their abilities to inhibit the human tumor necrosis factor alpha-induced (TNF-α) activation of NF-κB, using the Panomics NF-κB stable reporter cell line 293/NF-κB-luc. A series of 75 compounds was screened to identify substituted *trans*-stilbenes that were more active than resveratrol. Dose−response studies of the most active compounds were carried out to obtain IC_{50} values. Numerous compounds were identified that were more active than resveratrol, including compounds that were devoid of hydroxy groups and were 100-fold more potent than resveratrol. The substituted *trans*-stilbenes that were potent inhibitors of the activation of NFκB generally did not exhibit antioxidant activity. The results from screening were confirmed using BV-2 microglial cells where resveratrol and analogues were shown to inhibit LPS-induced COX-2 expression.

Introduction

The nuclear factor κB (NF-κB[a]) family of transcription factors in mammals consists of homo- and heterodimeric combinations of five related proteins (p50, p52, p65/RelA, c-Rel, and RelB) that have a marked influence on the expression of numerous genes involved in immunity and inflammation, as well as cellular stress responses, growth, and apoptosis. Diverse pathways activate NF-κB, and control of these pathways is increasingly viewed as an approach to chemotherapy in the many diseases that have an associated inflammatory component, including cancer, stroke, Alzheimer's disease, and diabetes.[1−10] Activation of NF-κB occurs through multiple pathways. The classical pathway is triggered by binding of proinflammatory cytokines (TNFα and IL-1) and of a number of pathogens to several different receptors in the TNF-receptor and Toll-like/IL-1 receptor superfamilies. This leads to recruitment to the plasma membrane and activation of the IκB-kinase complex (IKK) consisting of IKKα and IKKβ kinases, and the scaffold protein NEMO/ IKKγ, as well as a number of IKK-associated proteins. The main NF-κB that is activated in the classical pathway is the p50/p65 heterodimer that exists in the cytoplasm as a complex with inhibitory protein IκBα. Activation of IKK primarily through IKKβ results in phosphorylation of IκBα on Ser32 and Ser36, followed by polyubiquitination and degradation of IκBα by the 26S proteasome, allowing p50/ p65 to translocate to the nucleus.

Release of p50/p65 from IκBα also can be achieved by IKK-independent pathways triggered by DNA damage or oxidative stress that result in phosphorylation of IκBα on Ser residues other than Ser32 or Ser36, again leading to proteosomal degradation of IκBα. This signaling pathway involves a number of kinases including the MAP kinase p38 and casein kinase 2. There is also an oxidative stress pathway that phosphorylates IκBα on Tyr residues, leading to release of p50/p65 without proteosomal degradation of IκBα. Superimposed on the complex activation of p50/p65 is additional downstream regulation of the DNA-binding properties of p50/p65 through phosphorylation, acetylation, and peptidyl-prolyl isomerization. Mostly this occurs in p65 and provides multiple points for control of NF-κB activation in a cell-specific and environment-specific manner. A wide range of kinases can phosphorylate p50/p65, which appears essential for the transactivation potential of p50/p65. This includes phosphorylation at many different sites, especially in p65, which adds to the complex regulation of NF-κB.[4,10]

There are also alternative pathways to activation of NF-κB that result in formation of homo- or heterodimers other than p50/p65. A major alternative pathway, which is independent of IKKβ and NEMO, involves the IKKα homodimer whose activation is triggered by cytokines (other than TNFα), ligands such as CD40, and by certain viruses. This pathway requires recruitment of NF-κB-inducing kinase (NIK) with subsequent

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[a] Abbreviations: transcription factor nuclear factor kappaB (NF-κB); human tumor necrosis factor alpha (TNF-α).

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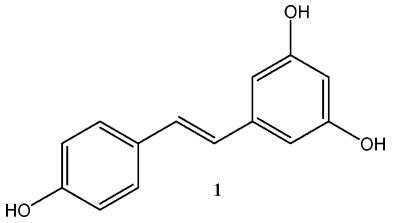
phosphorylation and activation of the IKKα homodimer. Activated IKKα phosphorylates p100, which is subsequently ubiquitinated and processed by the proteosome to p52. p52 and RelB then form a heterodimer that translocates to the nucleus. As with p50/p65, the p52/RelB heterodimer is further regulated by phosphorylation.[4]

A large number of compounds including natural products have been reported to inhibit activation of NF-κB at one or more sites in the complex pathways of activation.[11] This includes resveratrol (3,4′,5-trihydroxystilbene, **1**), a polyphenolic phyto-

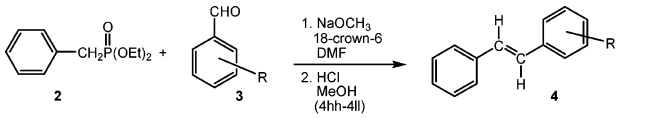


1

chemical that is found in numerous foods and is especially abundant in red wine. It has been proposed that the antioxidant activity of resveratrol is responsible for the French Paradox;[12−14] this relates to the low incidence of cardiovascular disease in a French population with high intake of saturated fat.[15] Both *trans* and *cis* isomers of resveratrol occur as phytochemicals, and both possess biological activities. Most studies of the biological activities of resveratrol and of synthetic stilbene analogues of resveratrol have focused on *trans* isomers. Resveratrol has been studied extensively in the context of carcinogenesis as a chemoprevention agent. All three stages of carcinogenesis, i.e., initiation, promotion and progression, have been reported to be inhibited by resveratrol.[16] Because resveratrol exhibits antioxidant activity, which is based upon its phenolic groups, much of the research on resveratrol and on polyphenolic analogues of resveratrol has focused on antioxidant properties.[17−21] In addition, the multiple biological activities reported for resveratrol, which in addition to its cardioprotective and anticarcinogenic activity also includes inhibition of platelet aggregation, modulation of lipoprotein metabolism, antiinflammatory, and vasorelaxing activities,[17,22−24] are often ascribed to the antioxidant properties of resveratrol. However, the oral bioavailability of resveratrol is low due to rapid metabolism, and the amount of resveratrol in dietary sources such as red wine is low compared to other polyphenols. Consequently, the circulating levels of resveratrol are low, suggesting that the direct antioxidant effects of resveratrol are unlikely to explain its biological activities.[12] Therefore, there has been extensive interest in the ability of resveratrol and other plant polyphenols to affect signaling pathways, including NF-κB.[25] Signaling through NF-κB has been shown to be involved in the ability of resveratrol to induce heme oxygenase-1,[26] inhibit phorbol ester-induced expression of COX-2,[27] inhibit TNFα-induced proliferation of smooth muscle cells,[28] enhance the radiosensitivity of lung cancer cells,[29] and inhibit nitric oxide and TNFα production by LPS-activated microglia.[30]

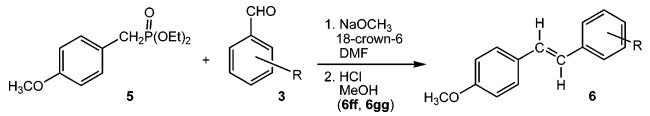
In the present study, we have screened a library of *trans*-stilbenes for their abilities to inhibit the TNFα-induced activation of NF-κB using the Panomics NF-κB stable reporter cell line 293/NF-κB-luc. This cell line was designed for screening inhibitors of the activation of NF-κB specifically in response to stimulation of the cells with TNFα, which therefore focuses on the classical activation pathway. This cell is a stable transfectant with a luciferase construct driven by an NF-κB-dependent promoter. In addition, the library was screened for

Scheme 1



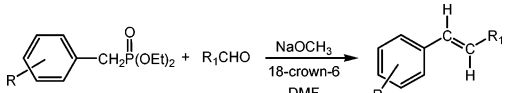
a: R = 3,4-OCH_3; b: R = 4-OCH_3; c: R = 4-Cl; d: R = 4-CH_3; e: R = 4-CN; f: R = 3,5-OCH_3; g: R = 2,6-OCH_3; h: R = 3-Cl; i: R = 3-CH_3; j: R = 2-Cl; k: R = 4-OCH_2CH_3; l: R = 4-OH; m: R = 4-F; n: R = 3-F; o: R = 2,3-OCH_3; p: R = 2-F; q: R = 3-OCH_3, 4-OH; r: R = 3-OCH_3; s: R = 3-OH, 4-OCH_3; t: R = 3-OH; u: R = 2,4-OCH_3; v: R = 2-CH_3; w: R = 3-CF_3; x: R = 4-CF_3; y: R = 2,5-OCH_3; z: R = 2-CF_3; aa: R = 2,4,6-CH_3; bb: R = 2-OCH_3; cc: R = 4-$N(CH_3)_2$; dd: R = 2,6-Cl; ee: R = 3,4-OH; ff: R = 3,4,5-OCH_3; gg: R = 2,3,4-OCH_3; hh: R = 4-OCH_2OCH_3; ii: R = 3-OCH_3, 4-OCH_2OCH_3; jj: R = 3-OCH_2OCH_3, 4-OCH_3; kk: R = 3-OCH_2OCH_3; ll: R = 3,4-OCH_2OCH_3

Scheme 2



a: R = 3,4-OCH_3; b: R = 4-OCH_3; c: R = 4-Cl; d: R = 4-CH_3; e: R = 4-CN; f: R = 3,5-OCH_3; g: R = 2,6-OCH_3; h: R = 3-Cl; i: R = 3-CH_3; j: R = 2-Cl; k: R = 4-OCH_2CH_3; l: R = 3,4,5-OCH_3; m: R = 4-F; n: R = 3-F; o: R = 2,3-OCH_3; p: R = 2-F; q: R = 2,4,5-OCH_3; r: R = 3-OCH_3; s: R = 2,3,4-OCH_3; t: R = 4-Br; u: R = 2,4-OCH_3; v: R = 2-CH_2CH_3, 3,4-OCH_3; w: R = 3-Br; x: R = 3,4-OH; y: R = 2,5-OCH_3; z: R = 2-CF_3; aa: R = 2,4,6-CH_3; bb: R = 2-OCH_3; cc: R = 2,3-OH; dd: R = 4-$N(CH_3)_2$; ee: R = 2,6-Cl; ff: R = 3,4-OCH_2OCH_3; gg: R = 2,3-OCH_2OCH_3

Scheme 3



a: R = H, R_1 = 3-C_5H_4N; b: R = H, R_1 = 4-C_5H_4N; c: R = H, R_1 = 2-C_5H_4N; d: R = H, R_1 = 2-C_4H_3S; e: R = H, R_1 = 2-$C_{10}H_7$; f: R = H, R_1 = 1-$C_{10}H_7$; g: R = OCH_3, R_1 = 2-$C_{10}H_7$; h: R = OCH_3, R_1 = 1-$C_{10}H_7$; i: R = OCH_3, R_1 = 2-C_4H_3S; j: R = OCH_3, R_1 = 2-C_5H_4N

antioxidant activities with two different assays. This allowed us to identify *trans*-stilbenes that were devoid of antioxidant activity and to demonstrate that some of these prevent the TNFα-induced activation of NF-κB. We report here that numerous *trans*-stilbenes were identified that are much more effective than resveratrol as inhibitors of the TNFα-induced activation of NF-κB.

Chemistry. The synthesis of a library of 75 (*E*)-stilbenes was accomplished as shown in Schemes 1−3. Initially our strategy for the construction of the *trans*-stilbene skeleton involved the reaction of an aromatic phosphonium ylide with substituted benzaldehydes.[31] This method proved to be unsatisfactory due to the formation of a mixture of *E* and *Z* isomers and the formation of triphenylphosphine oxide, which complicates the purification process. It is known that semistabilized ylides such as benzyl ylides give mixtures of isomers, which can be converted to *E* isomers by heating with a catalytic amount of iodine in heptane or toluene. In order to avoid these problems, Horner−Emmons−Wadsworth olefination chemistry was utilized as described by Lion et al.[32] Reaction of benzylphosphonic acid diethyl ester (**2**) with substituted benzaldehydes (**3a**−**k**, **3m**−**p**, **3r**, **3u**−**dd**, **3ff**, **3gg**) or methoxymethyl (MOM) hydroxyl substituted benzaldehydes (**3hh**−**3ll**) in DMF using sodium methoxide as the base in the presence of 18-crown-6 at 120 °C afforded 33 substituted stilbenes or methoxymethyl hydroxystilbenes (**4**) exclusively in the (*E*)-conformation (Scheme 1). There was no detectable *Z* isomer by ^{1}H NMR analysis. The diethylphosphoric acid byproduct is water soluble and was easily removed. In the case of the MOM protected benzaldehydes, (**4hh**−**4ll**), which were stable under the Horner−Emmons−Wadsworth conditions, the methoxymethyl protecting group was readily removed in a second step using hydrochloric acid to give the phenolic stilbene.

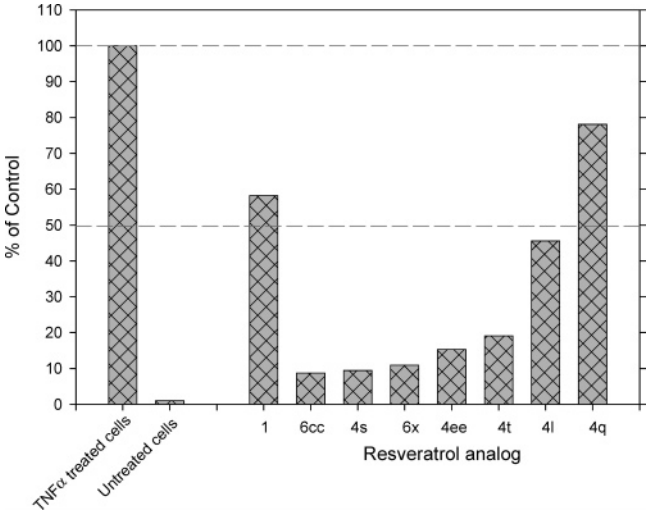


Figure 1. Inhibition of the TNFα-induced activation of NF-κB by resveratrol (**1**) and analogues of resveratrol (Scheme 1). All of the resveratrol analogues retained antioxidant activity.

Reaction of *p*-methoxybenzylphosphonic acid diethyl ester (**5**) with the appropriately substituted benzaldehydes (**3a**−**w**, **3y**−**bb**, **3dd**, **3ee**) or methoxymethyl hydroxyl substituted benzaldehydes (**3ff, 3gg**) under the same Horner−Emmons−Wadsworth conditions afforded 31 *trans*-stilbenes (**6**) having one anisole ring as shown in Scheme 2. The methoxymethyl protecting group on compounds **6ff** and **6gg** was readily removed using hydrochloric acid to give **6x** and **6cc**.

Scheme 3 shows the reaction of benzyl- or *p*-methoxybenzylphosphonic acid diethyl esters (**2** or **5**) with pyridyl, thienyl, and naphthyl aldehydes (**7**) under Horner−Emmons−Wadsworth conditions to afford 10 pyridyl, thienyl, and naphthyl *trans*-stilbenes (**8**).

Results

The first group of resveratrol analogues (Figure 1) retain hydroxy functional groups on one or both of the aromatic rings. Resveratrol and seven analogues were screened at 15 μM concentration. All of the analogues in this series retained antioxidant activity in the TRAP or FRAP assay. The *total radical-trapping antioxidant parameter* assay (TRAP assay) measures the ability of an analogue to react with the preformed radical monocation of 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\cdot+}$). The *ferric reducing/antioxidant power* assay (FRAP assay) measures the ability of an analogue to reduce a ferric tripyridyltriazine complex. Analogues **6cc** and **6x** were selected for further study. Analogues that also contained a methoxy functional group were the most active analogues in this series. Therefore, a separate series of substituted *trans*-stilbenes was synthesized that contained only methoxy groups.

Twenty three stilbenes in the methoxy-substituted series were screened and compared to resveratrol (Figure 2). Fourteen of the 23 are more active than resveratrol as inhibitors of TNFα-induced activation of NFκB. Stilbene **6r** was selected for additional study. None of the stilbenes in Figure 2 retained antioxidant activity. Clearly, antioxidant activity is not essential for the ability of these compounds to prevent the TNFα-induced activation of NF-κB.

Seventeen substituted *trans*-stilbenes were synthesized that contained a variety of substituents other than hydroxy or methoxy groups on one of the rings and no substituent on the other ring. Thirteen of these were more active than resveratrol as inhibitors of TNFα-induced activation of NF-κB in the preliminary screen (Figure 3). Stilbenes **4cc** and **4k** were selected for further study. Only **4cc** retained antioxidant activity.

Seventeen substituted *trans*-stilbenes were synthesized that contained a variety of functional groups on one ring, excluding hydroxy groups, and having a methoxy group on the other ring (Figure 4). Fifteen of these compounds are more active than resveratrol. Stilbenes **6p**, **6h**, **6n**, **6j**, **6i**, and **6d** were selected for further study.

Ten compounds were synthesized that contain one ring that is either a heterocyclic ring or a naphthalene ring (Figure 5). These compounds are only remotely related to resveratrol. Four of these compounds have a methoxy group on one ring, and a number of these compounds are more active than resveratrol. Stilbene **8e** was selected for further study.

Resveratrol and the 12 substituted *trans*-stilbenes that were selected for determination of IC_{50} values were analyzed in triplicate. IC_{50} values along with antioxidant activity and calculated ClogP values are summarized in Table 1. Several points are noteworthy. Some of the *trans*-stilbenes, such as

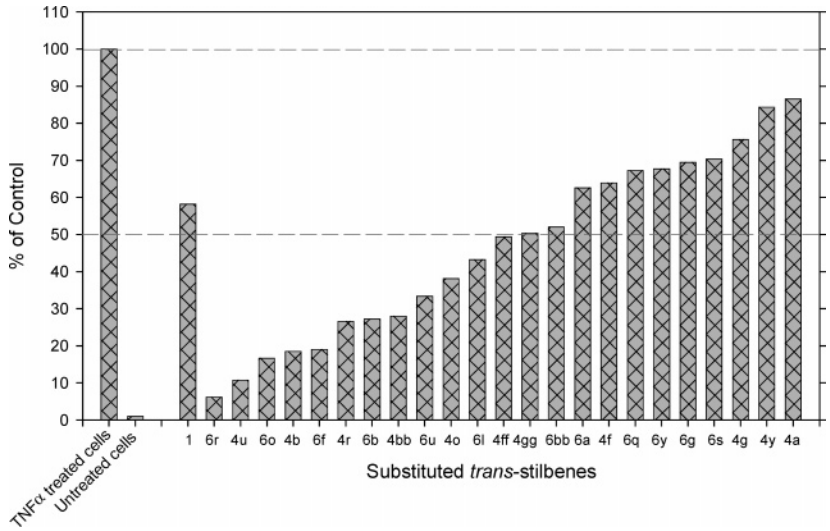


Figure 2. Inhibition of the TNFα-induced activation of NF-κB by substituted *trans*-stilbenes containing only methoxy substituents (Schemes 1 and 2).

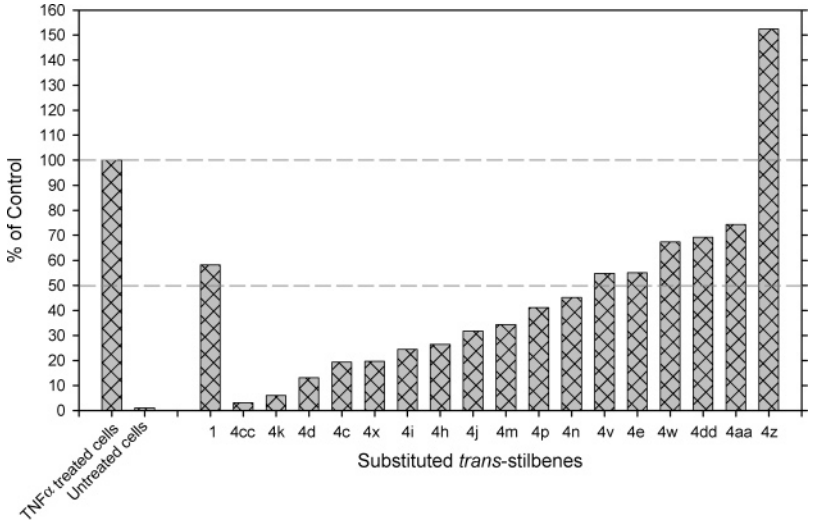


Figure 3. Inhibition of the TNFα-induced activation of NF-κB by substituted *trans*-stilbenes devoid of phenolic or methoxy groups.

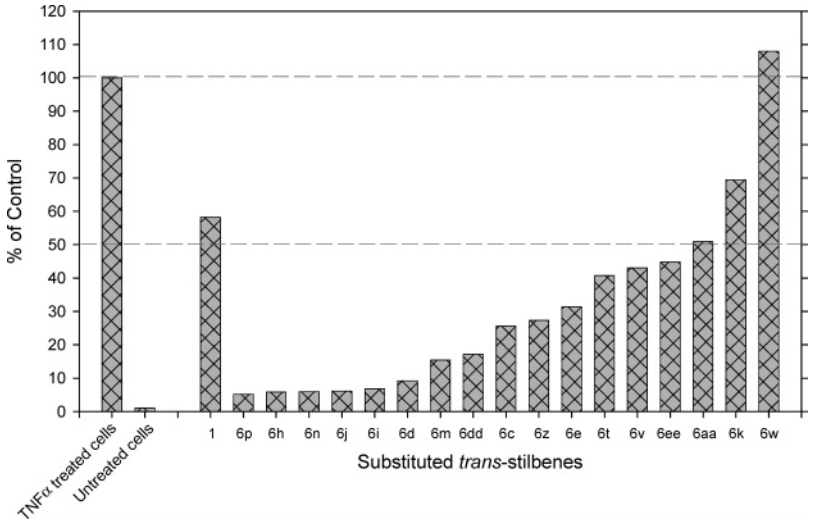


Figure 4. Inhibition of the TNFα-induced activation of NF-κB by a series of *trans*-stilbenes devoid of phenolic groups (Scheme 2).

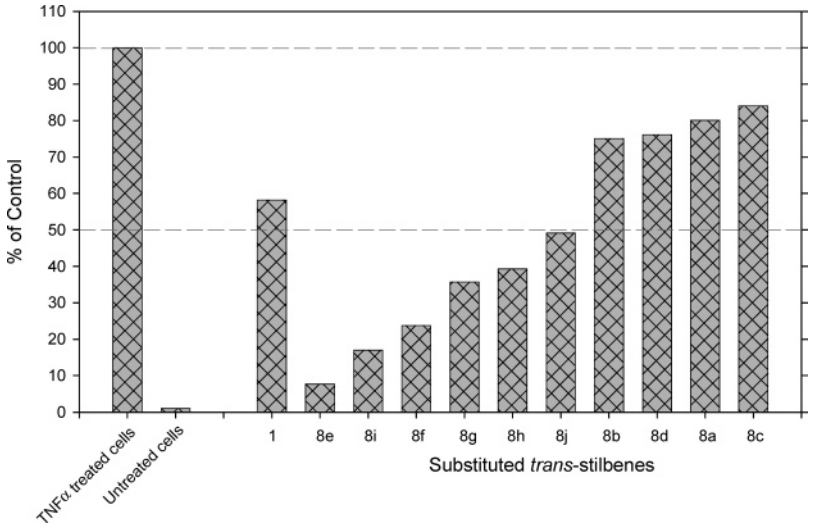
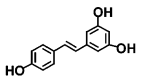
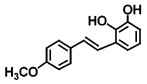
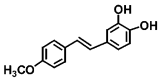
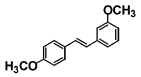
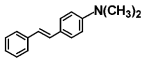
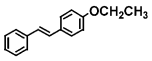
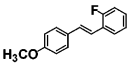
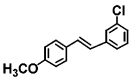
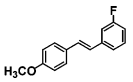
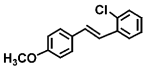
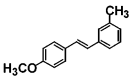
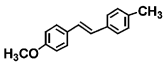
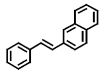


Figure 5. Inhibition of the TNFα-induced activation of NF-κB by analogues of *trans*-stilbenes (Scheme 3).

compounds **4cc** and **6p**, are more than 100-fold more potent than resveratrol. Modest changes in the nature of the ring substituent or its location can markedly affect activity. Most of the compounds in Table 1 do not retain antioxidant activity.

The toxicities of the 75 compounds evaluated in this study were determined. The initial screening that was carried out at 15μM concentrations of resveratrol or substituted *trans*-stilbenes involved exposure of the cells to TNFα and to inhibitor for 7

Table 1. IC_{50} Values of Resveratrol and Substituted *trans*-Stilbenes for Inhibition of the TNFα-Induced Activation of NF-κB

Number	Structure	IC_{50} (μM)	Anti-oxidant Activity TRAP	FRAP	CLogP
1 resveratrol		20 ± 3	+	+	2.833
6cc		0.5 ± 0.3	+	+	3.089
6x		0.6 ± 0.4	+	+	3.089
6r		0.6 ± 0.1	−	−	4.272
4cc		0.15 ± 0.1	+	+	4.599
4k		0.3 ± 0.03	−	−	4.882
6p		0.15 ± 0.1	−	−	4.496
6h		1.0 ± 0.1	−	−	5.066
6n		1.1 ± 0.6	−	−	4.496
6j		1.5 ± 0.03	−	−	5.066
6i		0.8 ± 0.2	−	−	4.852
6d		0.9 ± 0.1	−	−	4.852
8e		1.3 ± 0.3	−	−	5.608

hours. There was no apparent change in cell morphology. As a follow-up, the compounds in Table 1 were analyzed further by determination of cell viability, again after 7 h and with exposure to 15 μM concentrations. In all cases, there was no loss in cell viability compared to untreated controls.

To determine whether the effects of resveratrol and its analogues in inhibiting the activation of NF-κB extend beyond the cell line used for screening, resveratrol and analogues **4cc** and **6p** were compared using microglial BV-2 cells. This cell line has been shown to express COX-2 in response to LPS stimulation by an NF-κB-dependent pathway.[33] BV-2 cells stimulated with LPS showed a strong induction of COX-2 mRNA (Figure 6) that was markedly suppressed by 20 μM resveratrol and about 50% inhibited by 4 μM resveratrol. Analogue **4cc** and analogue **6p** at 0.15 μM concentrations were almost as effective as 4 μM resveratrol, consistent with the conclusions from Table 1 that these two analogues are more potent than resveratrol.

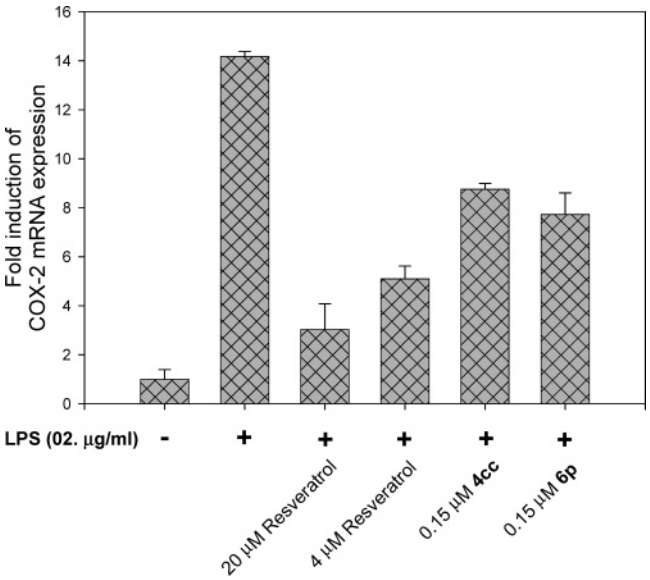
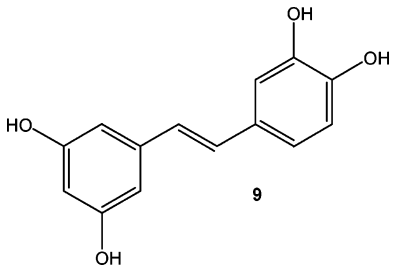


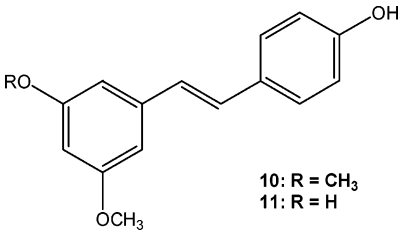
Figure 6. Inhibitory effects of resveratrol and analogues **4cc** and **6p** on LPS-induced expression of COX-2 mRNA in BV-2 microglial cells. Error bars represent standard deviations, $n = 3$.

Discussion

Resveratrol is one of several polyhydroxylated stilbene natural products with biological activity. Piceatannol (**9**), which is



present in the seeds of *Euphorbia lagascae*, is similar to resveratrol except for the presence of an additional hydroxyl functional group. Piceatannol exhibits antiinflammatory and antiproliferative activities[17] and induces apoptosis in lymphoma cells.[34] Piceatannol, like resveratrol, also inhibits TNF(α)-induced activation of NF-κB whereas stilbene itself is inactive.[35] Pterostilbene (**10**) and 3′-hydroxypterostilbene (**11**) are natural



analogues of resveratrol and piceatannol, respectively, that exhibit chemopreventive and apoptosis-inducing activities.[36,37] These two analogues as well as resveratrol itself show markedly different apoptosis-inducing activities against sensitive and resistant leukemia cells,[37] suggesting that minor structural changes in these hydroxylated stilbenes have major effects on biological activity.[21] However, all of these natural products retain one or more phenolic groups, which have been generally assumed to contribute both to antioxidant and to biological activities.

The *trans*-stilbenes in Figure 1 are analogues of resveratrol and related natural products. All of these analogues of resveratrol contain one or more hydroxy groups, and some of these analogues also contain methoxy groups. It is not surprising that this group of resveratrol analogues retains activity, in view of the reported activities for resveratrol, piceatannol, and related compounds.[21,35−37] The activities of the analogues in Figure 1 as inhibitors of the TNFα-induced activation of NF-κB, however, vary considerably; some compounds, such as analogues **6cc**, **4s**, and **6x**, are considerably more active than resveratrol. These three analogues contain one or two hydroxy groups and a single methoxy group. Therefore, it was of interest to evaluate *trans*-stilbenes that were devoid of hydroxy groups and would not be expected to exhibit antioxidant activity and to determine whether these compounds were still effective as inhibitors of the activation of NF-κB.

The 23 *trans*-stilbenes in Figure 2 contain one or more methoxy groups and include compounds with methoxy groups on both of the aromatic rings. None of these methoxy-substituted *trans*-stilbenes retained antioxidant activity in either the TRAP assay or the FRAP assay. Nevertheless, many of these compounds are more active than resveratrol as inhibitors of the TNFα-induced activation of NF-κB. This includes *trans*-stilbenes **6r**, **4u**, **6o**, **4b**, and **6f** that are especially active and contain one to three methoxy groups, either on one or on both rings and in different positions. Other compounds in this group, such as **6b** and **4o**, are isomers of **6r** but retained very little activity whereas **6r** is highly active.

Some of the most active *trans*-stilbenes contain substituents other than hydroxy or methoxy groups (Figures 3 and 4) and include compounds with substituents on one or both rings. *trans*-Stilbene **4cc** with a dimethylamino substituent in the 4-position and *trans*-stilbene **6p** with 2-fluoro and 4′-methoxy substituents were the most active of the 75 compounds included in this study; both **4cc** and **6p** showed IC_{50} values of 0.15 μM, which is >100-fold more potent than resveratrol. Compound **4cc** exhibits antioxidant activity in both the FRAP and TRAP assays, whereas compound **6p** does not exhibit antioxidant activity.

The actual target(s) whereby the most active substituted *trans*-stilbenes (Table 1) inhibit the TNFα-induced activation of NF-κB remains to be identified. Resveratrol has been shown to suppress the TNF(α)-induced phosphorylation and nuclear translocation of the p65 subunit of NF-κB.[38] Both IKKα and IKKβ are able to catalyze the phosphorylation of p65, although through different signaling pathways,[39] and are potential targets. Likewise, one or more of the kinases that activate IKK by phosphorylation, in response to TNFα or to the numerous other activators of NF-κB,[35] may be the targets.

Conclusions

We have demonstrated that the activation of NF-κB by TNFα can be effectively inhibited by a wide range of substituted *trans*-stilbenes, many of which do not contain hydroxyl functional groups and, therefore, are no longer analogues of resveratrol and related natural products. Compounds were identified that were devoid of antioxidant activity but were at least 100-fold more potent than resveratrol.

Experimental Section

Assay of the Antioxidant Activities of Resveratrol and Substituted *trans*-Stilbenes. The antioxidant activities of resveratrol and substituted *trans*-stilbenes were determined using two standard assays,[40] the TRAP assay[41] and the FRAP assay.[42] For the TRAP assay, 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) was reacted with potassium persulfate in the dark, overnight, to generate the colored $ABTS^{\cdot+}$ radical cation, which has an absorption maximum at 734 nm. The activities of resveratrol and the series of substituted *trans*-stilbenes were determined by their abilities to quench the color of the radical cation. For the FRAP assay, the ferric complex of 2,4,6-tripyridyl-*s*-triazine was prepared at acidic pH, and the antioxidant activities of resveratrol and the substituted *trans*-stilbenes were determined by their abilities to reduce the ferric complex to the ferrous complex, monitored by formation of the ferrous complex at 593 nm. In both colorimetric assays, the vitamin E analogue Trolox was used as a control.

Cell Assay. An NF-κB reporter stable cell line derived from human 293T embryonic kidney cells (293T/NF-κB-luc) (Panomics, Inc., Redwood City, CA) was grown in a humidified atmosphere at 37 °C in 5% CO_2/95% air. The cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM−high glucose containing 4 mM glutamine) supplemented with 10% fetal bovine serum (FBS), 1 mM sodium pyruvate, 100 units/mL penicillin, 100 μg/mL streptomycin, and 100 μg/mL hygromycin (Gibco/Invitrogen, Carlsbad, CA) to maintain cell selection. One day prior to treatment, the 293T/NFκB-luc cells were plated into 24-well cell culture plates (Costar, Cambridge, MA) at approximately 70% confluency in the above media without hygromycin. The following day cells were fed fresh media 1 h prior to treatment. Media with or without recombinant tumor necrosis factor alpha (TNFα) (R&D Biosciences/Clontech, Palo Alto, CA) was then applied to the cells at 20 ng/mL followed by immediate treatments with resveratrol or substituted *trans*-stilbene. The cells were placed again in a humidified atmosphere at 37 °C in 5% CO_2/95% air for 7 h. Plate wells were gently washed with phosphate buffered saline, pH 7.4, and lysed with 1x passive lysis buffer (Promega, Madison, WI). The subsequent lysates were analyzed with the Luciferase Assay System (Promega) utilizing a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA). The firefly luciferase relative light units were normalized to protein (mg/mL) with BCA Protein Assay Kit (Pierce, Rockford, IL) and standardized to percent of control (TNFα control).

For assays of cell viability, cells were treated similarly as above and with 15 μM substituted *trans*-stilbene. After being washed, cells were treated with 100 μL media and 20 μL CellTiter 96 AQ_{ueous} One Solution reagent for 1 h and then read at 490 nm with a Spectromax plate reader.

Inhibition of COX-2 Expression by Resveratrol and Analogs. Mouse microglial cells (BV-2), obtained from Dr. Paul M. Stemmer (Institute of Environmental Health Sciences, Wayne State University, Detroit, MI), were cultured in RPMI-1640 (Cellgro, Herndon, VA) supplemented with 10% FBS, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 μg/mL streptomycin sulfate, and 100 units/mL penicillin. Cells were grown on culture plates, pretreated with 1% gelatin for 30 min, at 37 °C and passaged twice weekly. BV-2 cells were activated with 0.2 μg/mL lipopolysaccharide (LPS) (Sigma, St. Louis, MO). Those cells that were treated with LPS were incubated in parallel with resveratrol or resveratrol analogues **4cc** or **6p** for 24 h at the indicated concentrations. Total RNA was purified using RNeasy (Qiagen, Valencia, CA) and converted to cDNA using TaqMan Reverse Transcriptase (Applied Biosystems, Branchburg, NJ). Cyclooxygenase-2 (COX-2) mRNA levels were measured using quantitative Real Time PCR analysis (qRT-PCR) of cDNA samples. Primers specific for COX-2 were designed to amplify a 132 base pair sequence flanking intron 7. Primer sequences for COX-2 were as follows: upstream, TGGGGTGATGAGCAACTATT; downstream, AAGGAGCTCTGGGTCAAACT. qRT-PCR was performed using ABsolute QPCR SYBR Green Mix (Fisher Scientific, Atlanta, GA) with the following cycling parameters: 1 cycle, 95 °C, 15 min; 40 cycles, 95 °C, 15 s, 60 °C, 1 min. β-Actin mRNA levels were quantitated using identical cycling conditions and used to normalize values obtained for COX-2 expression.

General Method for Synthesis of (*E*)-Stilbenes. To a solution of phosphonic acid diethyl ester (5 mmol) in 10 mL of dry DMF there was added sodium methoxide (10 mmol) and 18/6 crown ether (2 mmol). The resulting mixture was stirred at room temperature

for 5 min, and the appropriate aldehyde or hydroxybenzaldehyde methoxymethyl ether (6 mmol) dissolved in 5 mL of DMF was added dropwise at 0 °C. The mixture was stirred at room temperature for 1 h and then for 5 h at 120 °C. The reaction was quenched by pouring into 200 mL of water with stirring. Reactions that gave solids were filtered and recrystallized from hexane or ethanol. Reactions that gave oils were extracted into ether, and the ether layer was washed with water and saturated aq NaCl and dried ($MgSO_4$). Filtration and evaporation of the ether afforded oily solids that were purified by recrystallization or chromatography (hexane/ethyl acetate). Methoxymethyl protected hydroxystilbenes were heated in methanol containing 2 drops of concentrated hydrochloric acid to give hydroxystilbenes.[43]

(*E*)-2,6-Dimethoxystilbene (4g). mp 45−46 °C; ^{1}H NMR: δ 3.89 (s, 6H), 7.18 (m, 6H), 7.33 (t, 1H, J = 7.35 Hz), 7.45 (d, 1H, J = 16.68 Hz), 7.53 (d, 2H, J = 6.75 Hz). Exact mass calcd for $C_{16}H_{16}O_2$: 240.1550, observed (M + H) 241.1228.

(*E*)-3-Hydroxy-4-methoxystilbene (4s). mp 149−153 °C; ^{1}H NMR: δ 3.88 (s, 3H), 5.65 (s, 1H), 6.82 (d, 1H, J = 8.34 Hz), 6.97 (m, 3H), 7.15 (s, 1H, J = 1.98 Hz), 7.23 (m, 1H), 7.34 (t, 2H, J = 7.35 Hz), 7.48 (d, 2H, J = 7.15 Hz). Exact mass calcd for $C_{15}H_{14}O_2$: 226.0994, observed (M + H) 227.1072.

(*E*)-2-Trifluoromethylstilbene (4z). oil; ^{1}H NMR: δ 7.07 (d, 1H, J = 16.09 Hz), 7.36 (m, 5H), 7.52 (m, 3H), 7.65 (d, 1H, J = 7.75 Hz), 7.77 (d, 1H, J = 7.95 Hz). Exact mass calcd for $C_{15}H_{11}F_3$: 248.0813, observed (M + H) 249.0891.

(*E*)-2,6-Dichlorostilbene (4dd). oil; ^{1}H NMR: δ 7.13 (m, 2H), 7.35 (m, 6H), 7.54 (d, 2H, J = 7.54 Hz). Exact mass calcd for $C_{14}H_{10}Cl_2$: 298.1569, observed (M + H) 299.1647.

(*E*)-2,4′,6-Trimethoxystilbene (6g). mp 45−46 °C; ^{1}H NMR: δ 3.72 (s, 3H), 3.79 (s, 6H), 6.49 (d, 2H, J = 8.34 Hz), 6.79 (d, 2H, J = 8.74 Hz), 7.05 (t, 1H, J = 8.34 Hz), 7.24 (d, 1H, J = 16.68 Hz) 7.39 (d, 2H, J = 8.74 Hz), 7.45 (d, 1H, J = 16.48 Hz). Exact mass calcd for $C_{17}H_{18}O_3$: 270.1256 observed (M + H) 271.1334.

(*E*)-2,3,4,4′-Tetramethoxystilbene (6s). mp 124−125 °C; ^{1}H NMR: δ 3.81 (s, 3H), 3.86 (s, 3H), 3.89 (s, 6H), 6.68 (d, 1H, J = 8.74 Hz), 6.88 (d, 2H, J = 8.74 Hz), 6.96 (d, 1H, J = 16.68 Hz), 7.19 (d, 1H, J = 16.29 Hz), 7.27 (d, 1H, J = 8.74 Hz), 7.44 (d, 2H, J = 8.74 Hz). Exact mass calcd for $C_{18}H_{20}O_4$: 300.1362, observed (M + H) 301.1440.

(*E*)-2-Ethyl-3,4,4′-trimethoxystilbene (6v). mp 97−98 °C; ^{1}H NMR: δ 1.17 (t, 3H, J = 7.55 Hz), 2.80 (q, 2H, J = 7.54 Hz), 3.81 (s, 3H), 3.82 (s, 3H), 3.86 (s, 3H), 6.78 (m, 2H) 6.87 (m, 3H), 7.14 (d, 1H, J = 15.89 Hz), 7.31 (d, 1H, J = 8.54 Hz), 7.42 (d, 2H, J = 8.54 Hz); ^{13}C NMR: δ 15.4, 19.7, 55.4, 55.8, 60.9, 110.1, 114.1, 121.2, 124.1, 127.5, 128.4, 129.9, 130.8, 136.2, 146.8, 152.0, 159.0. Exact mass calcd for $C_{19}H_{22}O_3$: 298.1569, observed (M + H) 299.1647.

(*E*)-3-Bromo-4′-methoxystilbene (6w). mp 114−115 °C; ^{1}H NMR: δ 3.18 (s, 3H), 6.87 (m, 3H), 7.04 (d, 1H, J = 16.28 Hz), 7.18 (t, 1H, J = 7.75 Hz), 7.35 (m, 2H), 7.43 (d, 2H, J = 8.74 Hz), 7.63 (s, 1H). Exact mass calcd for $C_{15}H_{13}BrO$: 288.0150, observed (M + H) 289.0228.

(*E*)- 2-Trifluoromethyl-4′-methoxystilbene (6z). oil; ^{1}H NMR: δ 3.80 (s, 3H), 6.89 (d, 2H, J = 8.74 Hz), 7.01 (d, 1H, J = 16.10 Hz), 7.31 (m, 2H), 7.48 (m, 3H) 7.63 (d, 1H, J = 7.74 Hz), 7.74 (d, 1H, J = 7.75 Hz). Exact mass calcd for $C_{16}H_{13}F_3O$: 278.0918, observed (M + H) 279.0996.

(*E*)-4′-Methoxy-2,4,6-trimethylstilbene (6aa). mp 68−69 °C; ^{1}H NMR: δ 2.28 (s, 3H), 2.33 (s, 6H), 3.82 (s, 3H), 6.51 (d, 1H, J = 16.69 Hz), 6.91 (m, 5H), 7.42 (d, 2H, J = 8.73 Hz). Exact mass calcd for $C_{18}H_{20}O$: 252.1514, observed (M + H) 253.1592.

(*E*)-2,3-Dihydroxy-4′-methoxystilbene (6cc). mp 125−126 °C; ^{1}H NMR: δ 3.82 (s, 3H), 5.18 (s, 1H), 5.54 (s, 1H), 6.75 (m, 2H), 6.88 (d, 2H, J = 8.74 Hz), 7.07 (m, 2H), 7.19 (d, 1H, J = 16.29 Hz), 7.45 (d, 2H, J = 8.73 Hz). Exact mass calcd for $C_{15}H_{14}O_3$: 242.0943, observed (M + H) 243.1021.

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Supporting Information Available: General methods for synthesis of benzylphosphonic acid diethyl esters and MOM-protected hydroxybenzaldehydes and physical and spectroscopic data for compounds **4a**−**f**,**h**−**r**,**t**−**y**,**aa**−**cc**,**ee**−**gg**, **6a**−**f**,**h**−**r**,**t**,**u**,**x**,**y**,**bb**,**dd**,**ee**, and **8a**−**j**. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Yamamoto, Y.; Gaynor, R. B. Therapeutic potential of inhibition of the NF-kappaB pathway in the treatment of inflammation and cancer. *J. Clin. Invest.* **2001**, *107*, 135−142.
(2) Kim, H. J.; Hawke, N.; Baldwin, A. S. NF-κB and IKK as therapeutic targets in cancer. *Cell Death Different.* 2006, advanced online publication.
(3) Kaltschmidt, B.; Widera, D.; Kaltschmidt, C. Signaling via NF-κB in the nervous system. *Biochim. Biophys. Acta* **2005**, *1745*, 287−299.
(4) Viatour, P.; Merville, M-P.; Bours, V.; Chariot, A. Phosphorylation of NF-kappaB and IkappaB proteins: implications in cancer and inflammation. *Trends Biochem. Sci.* **2005**, *30*, 43−52.
(5) Kumar, A.; Takada, Y.; Boriek, A. M.; Aggarwal, B. B. Nuclear factor-κB: its role in health and disease. *J. Mol. Med.* **2004**, *82*, 434−448.
(6) Hiscott, J.; Kwon, H.; Genin, P. Hostile takeovers: viral appropriation of the NF-kappaB pathway. *J. Clin. Invest.* **2001**, *107*, 143−151.
(7) Barkett, M.; Gilmore, T. Control of apoptosis by Rel/NF-kappaB transcription factors. *Oncogene* **1999**, *18*, 6910−6924.
(8) Karin, M.; Greten, F. R. NF-κB: linking inflammation and immunity to cancer development and progression. *Nature Rev. Immunol.* **2005**, *5*, 749−759.
(9) Herrmann, O.; Baumann, B.; de Lorenzi, R.; Muhammad, S.; Zhang, W.; Kleesiek, J.; Malfertheiner, M.; Kohrmann, M.; Potrovita, I.; Maegele, I.; Beyer, C.; Burke, J. R.; Hasan, M. T.; Bujard, H.; Wirth, T.; Pasparakis, M.; Schwaninger, M. IKK mediates ischemia-induced neuronal death. *Nat. Med.* **2005**, *11*, 1322−1329.
(10) Schmitz, M. L.; Mattioli, I.; Buss, H.; Kracht, M. NF-κB: a multifaceted transcription factor regulated at several levels. *ChemBioChem* **2004**, *5*, 1348−1358.
(11) http://people.bu.edu/gilmore/nf-kb/inhibitors
(12) Dore, S. Unique properties of polyphenol stilbenes in the brain: more than direct antioxidant actions; gene/protein regulatory activity. *Neurosignals* **2005**, *14*, 61−70.
(13) Kundu, J. K.; Surh, Y-J. Molecular basis of chemoprevention by resveratrol: NF-κB and AP-1 as potential targets. *Mut. Res.* **2004**, *555*, 65−80.
(14) Shimizu, M.; Weinstein, B. Modulation of signal transduction by tea catechins and related phytochemicals. *Mut. Res.* **2005**, *591*, 147−160.
(15) Renaud, S.; de Lorgeril, M. Wine, platelets, and the French paradox for coronary heart disease. *Lancet* **1992**, *339*, 1523−1526.
(16) Jang, M.; Cai, L.; Udeani, G. O.; Slowing, K. V.; Thomas, C. F.; Beecher, C. W.; Fong, H. H.; Farnsworth, N. R.; Kinghorn, A. D.; Mehta, R. G.; Moon, R. C.; Pezzuto, J. M. Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. *Science* **1997**, *275*, 218−220.
(17) Ovesna, Z.; Horvathova-Kozics, K. Structure-activity relationship of *trans*-resveratrol and its analogs. *Neoplasma* **2005**, *52*, 450−455.
(18) Orallo, F. Comparative studies of the antioxidant effects of *cis*- and *trans*-resveratrol. *Curr. Med. Chem.* **2006**, *13*, 87−98.
(19) Lu, M.; Cai, Y. J.; Fang, J. G.; Zhou, Y. L.; Liu, Z. L.; Wu, L. M. Efficiency and structure-activity relationship of the antioxidant action of resveratrol and its analogs. *Pharmazie* **2002**, *57*, 474−478.
(20) Stojanovic, S.; Sprinz, H.; Brede, O. Efficiency and mechanism of the antioxidant action of *trans*-resveratrol and its analogues in the radical liposome oxidation. *Arch. Biochem. Biophys.* **2001**, *391*, 79−89.
(21) Stivala, L. A.; Savio, M.; Carafoli, F.; Perucca, P.; Bianchi, L.; Maga, G.; Forti, L.; Pagnoni, U. M.; Albini, A.; Prosperi, E.; Vannini, V. Specific structural determinants are responsible for the antioxidant activity and the cell cycle effects of resveratrol. *J. Biol. Chem.* **2001**, *276*, 22586−22594.
(22) Chung, M. I.; Teng, C. M.; Cheng, K. L.; Ko, F. N.; Lin, C. N. An antiplatelet principle of Veratrum formosanum. *Planta Med.* **1992**, *58*, 274−276.
(23) Fremont, L.; Gozzelino, M. T.; Linard, A. Response of plasma lipids to dietary cholesterol and wine polyphenols in rats fed polyunsaturated fat diets. *Lipids* **2000**, *35*, 991−999.
(24) Murias, M.; Handler, N.; Erker, T.; Pleban, K.; Ecker, G.; Saiko, P.; Szekeres, T.; Jager, W. Resveratrol analogues as selective cyclooxygenase-2 inhibitors: synthesis and structure-activity relationship. *Bioorg. Med. Chem.* **2004**, *12*, 5571−5578.

(25) Shay, N. F.; Banz, W. J. Regulation of gene transcription by botanicals: novel regulatory mechanisms. *Annu. Rev. Nutr.* **2005**, *25*, 297−315.
(26) Juan, S. H.; Cheng, T. H.; Lin, H. C.; Chu, Y. L.; Lee, W. S. Mechanism of concentration-dependent induction of heme oxygenase-1 by resveratrol in human aortic smooth muscle cells. *Biochem. Pharmacol.* **2005**, *69*, 41−48.
(27) Kundu, J. K.; Shin, Y. K.; Surh, Y. J. Resveratrol inhibits phorbol ester-induced expression of COX-2 and activation of NF-kappaB in mouse skin by blocking IkappaB activity. *Carcinogenesis* **2006**, Epub ahead of print.
(28) Lee, B.; Moon, S-K. Resveratrol inhibits TNF-α-induced proliferation and matrix metalloproteinase expression in human vascular smooth muscle cells. *J. Nutr.* **2005**, *135*, 2767−2773.
(29) Liao, H. F.; Kuo, C. D.; Yang, Y. C.; Lin, C. P.; Tai, H. C.; Chen, Y. Y.; Chen, Y. J. Resveratrol enhances radiosensitivity of human non-small cell lung cancer NCI-H838 cells accompanied by inhibition of nuclear factor-kappa B activation. *J. Radiat. Res. (Tokyo)* **2005**, *46*, 387−393.
(30) Bi, X. L.; Yang, J. Y.; Dong, Y. X.; Wang, J. M.; Cui, Y. H.; Ikeshima, T.; Zhao, Y. Q.; Wu, C. F. Resveratrol inhibits nitric oxide and TNF-alpha production by lipopolysaccharide-activated microglia. *Int. Immunopharmacol.* **2005**, *5*, 185−193.
(31) Bellucci, G.; Chiappe, C.; Lo, Moro, G. Crown ether catalyzed stereospecific synthesis of Z- and E-stilbenes by Wittig reaction in a solid-liquid two-phase system. *Tetrahedron Lett.* **1996**, *37*, 4225−4228.
(32) Lion, C. J.; Matthews, C. S.; Stevens, M. F. G.; Westwell, A. D. Synthesis, antitumor evaluation, and apoptosis-inducing activity of hydroxylated (*E*)-stilbenes. *J. Med. Chem.* **2005**, *48*, 1292−1295.
(33) Kang, G.; Kong, P. J.; Yuh, Y. J.; Lim, S. Y.; Yim, S. V.; Chun, W.; Kim, S. S. Curcumin suppresses lipopolysaccharide-induced cyclooxygenase-2 expression by inhibiting activator protein 1 and nuclear factor kappab bindings in BV2 microglial cells. *J. Pharmacol. Sci.* **2004**, *94*, 325−328.
(34) Wieder, T.; Prokop, A.; Bagci, B.; Essmann, F.; Bernicke, D.; Schulze-Osthoff, K.; Dorken, B.; Schmalz, H. G.; Daniel, P. T.; Henze, G. Piceatannol, a hydroxylated analog of the chemopreventive agent resveratrol, is a potent inducer of apoptosis in the lymphoma cell line BJAB and in primary, leukemic lymphoblasts. *Leukemia* **2001**, *15*, 1735−1742.
(35) Ashikawa, K.; Majumdar, S.; Banerjee, S.; Bharti, A. C.; Shishodia, S.; Aggarwal, B. B. Piceatannol inhibits TNF-induced NF-κB activation and NF-κB-mediated gene expression through suppression of IκBα kinase and p65 phosphorylation. *J. Immunol.* **2002**, *169*, 6490−6497.
(36) Rimando, A. M.; Cuendet, M.; Desmarchelier, C.; Mehta, R. G.; Pezzuto, J. M.; Duke, S. O. Cancer chemopreventive and antioxidant activities of pterostilbene, a naturally occurring analogue of resveratrol. *J. Agric. Food Chem.* **2002**, *50*, 3453−3457.
(37) Tolomeo, M.; Grimaudo, S.; Di, Cristina, A.; Roberti, M.; Pizzirani, D.; Meli, M.; Dusonchet, L.; Gebbia, N.; Abbadessa, V.; Crosta, L.; Barucchello, R.; Grisolia, G.; Invidiata, F.; Simoni, D. Pterostilbene and 3′-hydroxypterostilbene are effective apoptosis-inducing agents in MDR and BCR-ABL-expressing leukemia cells. *Int. J. Biochem. Cell. Biol.* **2005**, *37*, 1709−1726.
(38) Mann, S. K.; Mukhopadhyay, A.; Aggarwal, B. B. Resveratrol suppresses TNF-induced activation of nuclear transcription factors NF-κB, activator protein-1, and apoptosis: potential role of reactive oxygen intermediates and lipid peroxidation. *J. Immunol.* **2000**, *164*, 6509−6519.
(39) Sizemore, N.; Lerner, N.; Dombrowski, N.; Sakurai, H.; Stark, G. R. Distinct roles of the IκB kinase α and β in liberating nuclear factor κB (NF-κB) from IκB and in phosphorylating the p65 subunit of NF-κB. *J. Biol. Chem.* **2002**, *277*, 3863−3869.
(40) Schlesier, K.; Harwat, M.; Bohm, V.; Bitsch, R. Assessment of antioxidant activity by using different in vitro methods. *Free Rad. Res.* **2002**, *36*, 177−187.
(41) Re, R.; Pellegrini, N.; Proteggente, A.; Pannala, A.; Yang, M.; Rice-Evans, C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Rad. Biol. Med.* **1999**, *26*, 1231−1237.
(42) Benzie, I. F.; Strain, J. J. Ferric reducing/antioxidant power assay: direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentrations. *Methods Enzymol.* **1999**, *299*, 15−27.
(43) Begum, S. D.; Parthasarathi, J. A convenient synthesis of homobutein. *Ind. J. Chem. Sect. B* **1988**, *27B*, 464.

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