

RESEARCH ARTICLE

Resveratrol derivatives as promising chemopreventive agents with improved potency and selectivity

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Scope: Despite scores of investigations, the actual impact of resveratrol (3,5,4'-trihydroxy-*trans*-stilbene) on human health, as a dietary component or supplement, remains moot. This is due to many factors, such as relatively low potency, pleiotropic mechanisms, and rapid metabolism. Nonetheless, as a promiscuous molecule that interacts with numerous targets, resveratrol can be viewed as a scaffold for designing structural relatives potentially capable of mediating more intense responses with greater mechanistic stringency.

Methods and results: We currently report the synthesis and biological evaluation of 92 stilbene analogs. The compounds were tested with *in vitro* assays for activation of quinone reductase 1, inhibition of quinone reductase 2, nitric oxide production, aromatase, NFκB, 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced ornithine decarboxylase, or cyclooxygenase-1 and -2, quenching of 2,2-diphenyl-1-picrylhydrazyl free radical, interaction with estrogen receptors, and as antiproliferative agents. Several compounds were found to mediate responses with much greater potency than resveratrol; some mediated pleiotropic responses, as is the case with the parent molecule, but others were highly specific or totally inactive. When administered to rats, higher serum concentrations and greater stability was demonstrated with prototype lead molecules.

Conclusion: Owing to structural simplicity, facile syntheses are available for large-scale production. These data support the promise of more advanced development of novel resveratrol derivatives as drug entities.

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1 Introduction

As a natural product, resveratrol (3,5,4'-trihydroxy-*trans*-stilbene; compound 1) is a phytoalexin produced by plant

species in response to environmental stress or pathogenic attack. This compound was first reported by our group as a candidate for cancer chemoprevention [1] and extensive studies have followed [2, 3]. Resveratrol demonstrates a

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Abbreviations: AhR, aryl hydrocarbon receptor; AP, apicalBL, basolateral; COX-1, cyclooxygenase-1; COX-2, cyclooxygenase-2;

CYP, cytochrome; DPPH, 2,2-diphenyl-1-picrylhydrazyl; ER, estrogen receptor; HBSS, Hank's balanced salt solution; iNOS, inducible nitric oxide synthase; IR, induction ratio; LPS, lipopolysaccharide; ODC, ornithine decarboxylase; QR1, quinone reductase 1; QR2, quinone reductase 2; SAR, structure-activity relationship; SRM, selected reaction monitoring; TEER, transepithelial electrical resistance; TPCK, *N*-tosyl-L-phenylalaninyl-chlormethylketone; TPA, 12-*O*-tetradecanoylphorbol-13-acetate

broad spectrum of biological activities potentially capable of inhibiting carcinogenesis at the stages of initiation, promotion, and progression. Examples include down-regulation of the synthesis and release of proinflammatory mediators, inhibition of activated immune cells, aromatase, inducible nitric oxide synthase (iNOS), cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2), and effects on NF κ B [3]. Activation of NAD(P)H:quinone reductase 1 (QR1), a well-established mechanism for chemoprevention that correlates with the induction of other phase II detoxifying enzymes, is also facilitated by resveratrol [4–6]. The molecule is unusually promiscuous [7]. Although some high-affinity targets exist, such as quinone reductase 2 (QR2) [8], it does not appear likely that resveratrol functions through one specific mechanism. Considering the structural simplicity of this stilbene, the intensity of research publications is phenomenal, indicating that the molecule holds great potential [9–13].

In vivo studies suggest resveratrol is relatively safe and can be used for the prevention and therapy of cancer [13]. Studies with human beings suggest a daily dosage of 1–5 g may be acceptable [14]. Obviously, dosage levels in this range are not available through normal dietary consumption, so resveratrol is evolving more as a natural product drug than a natural dietary component that may affect human health. As such, it seems logical to investigate structural derivatives that may show greater efficacy and improved biophysical characteristics.

We currently report the synthesis and biological evaluation of a library of resveratrol derivatives. The derivatives include compounds differing in the number, position, and type of substituents, and the presence or absence of stilbenic double bond. Sulfate derivatives were tested as well, since serum concentrations of these metabolites are higher than those of resveratrol following treatment with the parent compound. A battery of in vitro assays was used to monitor the activity of all designed derivatives, and some preliminary absorption and metabolism studies have been performed with promising leads. Structure–activity relationships are beginning to emerge. In sum, based on information that has been gleaned from the extensive work that has been performed with resveratrol, it is relatively clear that derivatives with greater potency and specificity can be developed.

1.1 Background on resveratrol

Resveratrol, a naturally occurring polyphenol, is a phytoalexin found in grapes, mulberries, cranberries, blueberries, peanuts, and chocolate [1, 15, 16]. It is produced by these plants as a response to stress, such as a bacterial or fungal infection [17]. Resveratrol is produced from two precursor molecules, malonyl-coenzyme A and *p*-coumaroyl-coenzyme A, by the enzyme stilbene synthase [18]. The compound was first purified from white

hellebore in 1939 by Takaoka [19]. In 1963, it was found to be a component of Japanese ironwood, which is currently used as the source of many resveratrol supplements [20]. The consumption of wine entered the mainstream media in 1992, when it was touted as the explanation for the “French paradox” because of biological activities that improved cardiovascular health [21]. The lower incidence of cardiovascular diseases among the French people, in spite of their diet high in saturated fats had been reported [22, 23], and epidemiological studies linked moderate consumption of red wine with a lower incidence of heart disease. A link to resveratrol, in particular, was established later, when the cancer chemopreventive potential was described [1]. Since grape skins are included in the fermentation of red wine, it typically contains a significantly higher amount of resveratrol (2–40 μ M) [24] than white wine. Nonetheless, resveratrol concentration varies with the type of red wine, with Pinot noir sometimes containing the highest level of resveratrol [25]. The inverse correlation with heart disease was further strengthened when resveratrol was shown to decrease serum cholesterol [26], promote vasorelaxation [27, 28], suppress atherosclerosis [29], and inhibit platelet aggregation [30].

In the area of chemoprevention, following our observation of the potential of resveratrol to block 7,12-dimethylbenz(*a*)anthracene (DMBA)-induced tumorigenesis and various stages of carcinogenesis [1], a great number of studies have been performed. It has been shown to act through varied mechanisms, including the regulation of cell cycle progression [31], apoptosis [32, 33], inhibition of tumor invasion and angiogenesis [34], prevention of inflammation [35], activation of adenosine monophosphate-activated protein kinase (AMPK) [36], scavenging of reactive oxygen species, [37], and modulation of NF κ B [38, 39]. This activation of multiple anti-cancer pathways (pleiotropism) can be viewed as an attractive feature of resveratrol, since it may help to overcome drug resistance [40, 41]. It has been suggested that resveratrol is capable of mediating a great number of other biological responses relevant to human health [9], such as protection against viral infections [42], caloric restriction mimicry [43], as well as effects on longevity [44].

Resveratrol is readily absorbed in humans and rapidly metabolized into its sulfate and glucuronide conjugates [45]. In addition, it is well tolerated and does not appear to be toxic in humans [14, 45]. However, pharmacokinetic studies have shown that the concentrations necessary to mediate activity observed using in vitro experiments cannot be achieved in animals [46], so identification of derivatives with greater bioavailability is desirable. Isomerization of the double bond that links the two phenolic rings of resveratrol creates two possible geometrical isomers. The *trans* form is more common in nature due to thermodynamic factors [47]. Much less is known about the pharmacological effects of *cis*-resveratrol [48].

1.2 Pleiotropic effect of resveratrol

Carcinogenesis occurs over a long period of time through consecutive stages [49] with alterations of phenotypic, cellular, genotypic, molecular, and drug-related biomarkers [50]. Resveratrol has been reported to prevent or inhibit carcinogenesis by acting on multiple targets at all stages.

1.2.1 Antioxidant activity of resveratrol

Oxidative damage in cells has been implicated as a causative factor in many diseases including cancer and Alzheimer's, and has an impact on the aging process [51]. Resveratrol has demonstrated antioxidant potential by promoting various enzymes. We have shown that resveratrol can inhibit 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced free radical formation with cultured HL-60 cells. Also, resveratrol exhibited 2,2-diphenyl-1-picrylhydrazyl (DPPH) free-radical scavenging activity ($IC_{50} = 94.6 \mu\text{g/mL}$) and hypoxanthine/xanthine oxidase enzyme inhibitory activity ($IC_{50} = 59.1 \mu\text{g/mL}$) [52, 53]. Resveratrol suppresses the formation of super-oxide ($O_2^{\cdot-}$) and H_2O_2 produced by LPS or TPA-stimulated macrophages [54]. The compound inhibits H_2O_2 -induced DNA damage in human lymphocytes by increasing glutathione levels, and induction of glutathione peroxidase, glutathione reductase, and glutathione *S*-transferase [55].

1.2.2 Inhibition of inflammatory enzymes

It has been reported that chronic inflammation can lead to various cancers [56]. Therefore, inhibition of inflammation is a promising approach for cancer chemoprevention. Resveratrol inhibits the activity of COX-1 [1] and COX-2 as reflected in decreased levels of prostaglandin E_2 ($IC_{50} = 32.2 \mu\text{M}$) [57], suppresses iNOS in activated macrophages [58], and TNF receptor associated factor-binding kinase 1 in Toll-interleukin-1 (TIR) domain-containing adaptor inducing interferon-beta signaling in the TLR3 and TLR4 inflammatory pathway [59]. In TPA-induced mouse skin, resveratrol significantly inhibited COX-2 expression and NF κ B activation by inhibiting the activity of I κ B kinase [60].

1.2.3 Inhibition of cytochrome P450

Cytochrome (CYP) P450 isozymes are a large family of enzymes involved in the metabolism of xenobiotics [53]. Transcription of CYP1A1 is induced by the aryl hydrocarbon receptor (AhR). The aberrant expression of AhR has been reported in breast and prostate cancers [61]. Resveratrol inhibited TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin)-induced CYP1A1 expression by decreasing binding of AhR to the promoter region of CYP1A1 [62]. CYP1B1 was reported to

function as an estrogen 4-hydroxylase [63] and activate many carcinogens including estrogens. Resveratrol exhibited competitive inhibition of human CYP1A1 as well as non-competitive inhibition of human CYP1B1 [64], and inhibited aromatase enzyme (CYP19) activity ($IC_{50} = 12.8 \mu\text{M}$) [65].

1.2.4 Protein kinase C

Many studies have been performed to understand the function of protein kinase C in carcinogenesis and to find inhibitors for cancer treatment or prevention [66]. Resveratrol has been reported to inhibit TPA-mediated induction of protein kinase C [67].

1.2.5 Ornithine decarboxylase

Ornithine decarboxylase (ODC) is up-regulated in various tumor cells. Resveratrol significantly decreased the ODC activity in Caco-2 cells [68], and blocked *N*-nitrosodiethylamine-induced cytosolic ODC levels in the mice liver and lungs [69].

1.2.6 QR1 and QR2

Phase II enzymes are known to detoxify carcinogens, including aldehyde reductase, glutathione reductase, epoxide hydrolase, glutathione *S*-transferase, QR1, and Uridine 5'-siphosphate-glucuronosyltransferases [70]. Resveratrol induces QR1 activity with a concentration required to double activity (CD) of $21 \mu\text{M}$ in cultured mouse hepatoma (Hepa 1c1c7) cells [71]. Resveratrol is also a potent inhibitor of QR2, a cytosolic enzyme that catalyzes the reduction of quinones. It is hypothesized that inhibition of QR2 may lead to protection of cells against reactive species that are causing cellular damage [8].

1.2.7 Modulation of cancer cell cycle and apoptosis

Cell cycle mediators such as cyclins, cyclin-dependent kinases, and cyclin-dependent kinase inhibitors are involved in the deregulated cell cycle progression of cancer cells [72]. Resveratrol arrests various phases of the cell cycle, depending on cell types [73], and can induce apoptosis via enhancement of death receptor signaling [32, 74].

1.2.8 Absorption and metabolism

A notable characteristic of resveratrol is its relative lack of potency. Many potential health benefits have been suggested, but considerable controversy exists as to whether it is an active molecule *in vivo* [75]. Resveratrol is efficiently absor-

bed on oral administration and rapidly metabolized to its 3- and 4'-O-sulfate, and 3-O-glucuronide conjugates [76–78]. After a dietarily relevant 25 mg oral dose of resveratrol, the plasma concentration of resveratrol is only in the nanomolar range compared with the micromolar range of its metabolites [75, 77, 79]. Based on resveratrol metabolism studies, it is reasonable to suggest the in vitro data obtained using high concentrations of resveratrol lack direct in vivo relevance. Although large amounts of resveratrol can be administered, there is merit in searching for analogs with significantly greater potency, thereby reducing the need for large dosages and allowing comparisons with known modulators [80].

2 Materials and methods

2.1 Preparation of compounds

Syntheses of the resveratrol derivatives 2–93 have been reported previously [8].

2.2 Aromatase assay

Aromatase activity was assayed as previously reported [81]. Briefly, test compounds at final concentration of 50 μ M were preincubated with Nicotinamide adenine dinucleotide phosphate-regenerating system before the enzyme and substrate mixture were added, and the plate was incubated at 37°C before quenching with NaOH. Fluorescence was measured at 485 nm (excitation) and 530 nm (emission). IC₅₀ values and dose–response curves were based on two independent experiments performed in duplicate using five concentrations of test substance. Naringenin (IC₅₀ = 0.23 μ M) was used as a positive control.

2.3 NF κ B luciferase assay

Stably transfected human embryonic kidney cells 293 were used for monitoring any changes occurring along the NF κ B pathway [82] with tumor necrosis factor (TNF)- α (1 nM) as activator. After incubation with tested compounds, cells were lysed in reporter lysis buffer, and a luciferase assay was performed using the Luc assay system from Promega (Madison, WI, USA). Luminescence was detected in a LUMIstar Galaxy BMG luminometer. Data for NF κ B activity are expressed as IC₅₀ values. As a positive control, two NF κ B inhibitors were used: *N*-tosyl-L-phenylalaninyl-chlormethylketone (TPCK), IC₅₀ = 3.8 μ M and BAY-11, IC₅₀ = 2.0 μ M.

2.4 QR1 assay

QR1 activity was assessed using Hepa 1c1c7 murine hepatoma cells as previously described [83]. Test compounds

were added to a final concentration of 50 μ M, and QR activity was measured as a function of the NADPH-dependent menadiol-mediated reduction of 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide to a blue formazan. The induction ratio (IR) of QR activity represents the specific enzyme activity of agent-treated cells compared with a DMSO-treated control. The concentration to double activity (CD) was determined through a dose–response assay for active substances (IR > 2). The chemopreventive index (CI) is calculated as IC₅₀/CD. 4'-Bromoflavone (CD = 0.01 μ M) was used as a positive control.

2.5 QR2 assay

The QR2 assay was performed according the procedure described by Sun et al. [8]. Briefly, the assay mixture contained 12 nM QR2, 17.5 μ M NMeH, and 200 μ M 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide in a reaction buffer containing 100 mM NaCl, 50 mM Tris, and 0.1% Triton-X100 (Fisher Biotech 93004). All reactions were initiated by the addition of QR2. Activity of QR2 was determined using a SpectraMax Plus 384 UV–vis microplate reader by monitoring the increase in absorbance at 612 nm. IC₅₀ values were determined using the same assay conditions except that inhibitor concentrations were varied from 0.2 to 100 μ M. The percent inhibition data were then plotted as a function of inhibitor concentration, [I], and the data were then fit to the following equation: %I = %I_{max}/[(1 + [I]/IC₅₀)] using nonlinear regression describing a simple binding isotherm. All data were globally fit to the equation using the Enzyme Kinetics Module of the program SIGMAPLOT from SPSS Scientific (Chicago, IL, USA).

2.6 Measurement of nitric oxide production in LPS-stimulated macrophages

RAW 264.7 cells were treated with various concentrations of compounds followed by 1 μ g/mL of LPS. Nitrite in cultured media, a stable end product of NO, was estimated by the addition of the Griess reagent and the absorbance was measured at 540 nm. A standard curve was created by using known concentrations of sodium nitrite to convert the absorbance to the concentration of nitrite in cultured media [84].

2.7 Determination of TPA-induced ODC activity

Human epithelial urinary bladder cells T-24 were incubated in the absence of serum; resveratrol derivatives were added in duplicate before the induction of ODC activity with TPA. ODC activity was assayed by measuring the release of [¹⁴C]CO₂ from L-[1-¹⁴C]-ornithine HCl using a Beckman Coulter LS 6500 counter. IC₅₀ values were generated from

the results of five serial dilutions of tested derivatives in duplicate. Standard inhibitors of ODC activity include apigenin, $IC_{50} = 6.0 \mu M$, menadione, $IC_{50} = 8.3 \mu M$, and deguelin, $IC_{50} = 0.1 \mu M$ [85].

2.8 DPPH radical scavenging assay

To evaluate antioxidant capacity, DPPH free-radical scavenging was performed according to the method described by Lee et al. [52]. The absorbance was measured at 515 nm. The DPPH radical scavenging activity of each sample was evaluated by calculating percentage of inhibition as follows: $\%inhibition = (1 - Abs_{sample}/Abs_{control}) \times 100$.

2.9 COX-1 and -2 assays

Assays were conducted in a 96-well format [86] with analyses performed as follows. Negative-ion electrospray tandem mass spectro-metric measurement of PGE_2 was carried out using a Shimadzu (Columbia, MD, USA) LCMS-8030 triple quadrupole mass spectrometer equipped with a Shimadzu Nexera UHPLC system. A Waters (Milford, MA, USA) Acquity UPLC[®] BEH C_{18} column (2.1 mm \times 50 mm, 1.7 μm) was used for LC separation with a 0.5 min isocratic mobile phase consisting of ACN/aqueous 0.1% formic acid (40:60, v/v) at a flow rate of 1.2 mL/min. The deprotonated molecules of m/z 351 and 355 corresponding to PGE_2 and the surrogate standard d_4 - PGE_2 , respectively, and the abundant product ions of m/z 271 and 275, were measured using selected reaction monitoring (SRM).

2.10 Binding to human estrogen receptors using ultrafiltration LC-MS

An equimolar mixture of resveratrol analogs (0.2 μM each) was incubated for 2 h at room temperature with 100 pmol estrogen receptor (ER)- α or ER- β (human recombinant ER- α and ER- β produced using recombinant baculovirus-infected insect cells; Invitrogen, Carlsbad, CA, USA) in binding buffer consisting of 50 mM Tris-HCl (pH 7.4), 10% glycerol, 50 mM KCl and 1 mM EDTA in a total volume of 150 μL . Identical negative control incubations in which ER was omitted were used to correct for nonspecific binding of the compounds to the ultrafiltration membrane. 8-Prenylnaringenin, a potent phytoestrogen, was used as a positive control. After incubation, each mixture was filtered through a Microcon YM-30 centrifugal filter by centrifugation at $10\,000 \times g$ for 10 min at 4°C. The filter was washed three times by centrifugation with 150 μL aliquots of ammonium acetate buffer (pH 7.4) to remove the unbound compounds. The bound ligands were released by adding 400 μL of methanol/water (90:10, v/v) followed by centrifugation at $10\,000 \times g$ for 10 min. The ultrafiltrate was evaporated to

dryness under a stream of dry nitrogen and then reconstituted in 60 μL of methanol/water (50:50; v/v). Naringenin was added as an internal standard to normalize the ionization efficiency between experiments and controls. Aliquots (10 μL) were analyzed using LC-MS, which consisted of a Shimadzu LC-20AD HPLC coupled to a Shimadzu IT-TOF mass spectrometer. HPLC separation was carried out using a Waters Xterra MS C_{18} (3.5 μm , 2.1 \times 100 mm) HPLC column.

2.11 Caco-2 cell monolayer permeability and plasma protein binding

2.11.1 Materials

The Caco-2 cell line, Eagle's minimum essential medium, and fetal bovine serum were purchased from the American Type Culture Collection (Rockville, MD, USA). Trypsin-EDTA (0.25%), Hank's balanced salt solution (HBSS), HEPES buffer solution, PBS, penicillin, and streptomycin were purchased from Life Technology (Grand Island, NY, USA). Transwell polycarbonate inserts (24 mm diameter, 4.71 cm² surface, 0.4 μm pore size) were purchased from Corning Costar (Cambridge, MA, USA). Transepithelial electrical resistance (TEER) was measured using a Millicell-ERS instrument from Millipore (Bedford, MA, USA). Pooled human plasma was purchased from Innovative Research (Novi, MI, USA), and rapid equilibrium dialysis devices were purchased from Pierce Biotechnology (Rockford, IL, USA).

2.11.2 Caco-2 cell monolayer model assay

Caco-2 cells were cultured in Eagle's minimum essential medium with 10% fetal bovine serum, 100 units/mL penicillin and 100 $\mu g/mL$ streptomycin in a humidified atmosphere of 5% CO₂ and 95% air at 37°C. When the cells (passage numbers 10–12) had reached approximately 70–80% confluence, they were removed using 0.25% trypsin/EDTA, seeded onto polycarbonate membranes fitted into Transwell 6-well plates (24 mm diameter, 4.71 cm² surface area, 0.4 μm pore size) at a density of 2×10^5 cells/insert and cultured until late confluence. The cell culture medium was changed every other day after seeding. The integrity of each monolayer of differentiated cells was monitored by measuring the TEER. Only monolayers with TEER values $> 300 \Omega/cm^2$ were utilized.

In preparation for the Caco-2 cell monolayer assay, the cell culture medium was removed from both the apical (AP) and basolateral (BL) chambers. The cells were washed three times with PBS (pH 7.4) and pre-incubated with HBSS containing 20 mM HEPES, pH 7.4 for 30 min at 37°C on a shaker bath at 50 rpm. A 20 mM stock solution of resveratrol derivatives in DMSO was diluted to

different concentrations in HBSS/HEPES buffer. The test solutions were added either to the AP side (for AP→BL measurement) or to the BL side (BL→AP measurement) of the monolayers, and blank HBSS/HEPES buffer was added to the other side. As control experiments, the low permeability standard sucrose (50 μ M) and the high permeability standard propranolol (10 μ M) were added to the AP chambers of parallel incubations. Aliquots from the recipient compartments were collected at 10, 20, 30, 40, and 60 min and then analyzed using LC-MS-MS. In some experiments, the effects of the p-glycoprotein inhibitor verapamil (50 μ M) were investigated. All experiments were carried out in triplicate.

2.11.3 Binding to human plasma proteins

A rapid equilibrium device from Pierce Biotechnology was used as directed by the manufacturer for the determination of binding of tested derivatives to human plasma proteins. Briefly, 200 μ L of 10 μ M compound in pooled human plasma was pipetted into the rapid equilibrium dialysis device sample chamber. The buffer chamber was then filled with 350 μ L PBS consisting of 100 mM sodium phosphate and 150 mM sodium chloride. The device was sealed and placed in an orbital shaker at 37°C set at 200 rpm for 5 h. After dialysis, 100 μ L aliquots were removed from the buffer and plasma chambers and mixed with 100 μ L of plasma or PBS, respectively. Proteins were precipitated by addition of 600 μ L of ice-cold 90:10 v/v ACN/water containing 0.1% formic acid followed by incubation on ice for 30 min. After centrifugation for 15 min at 14 000 \times g, the supernatants were removed, evaporated to dryness, and then reconstituted in 100 μ L of HPLC mobile phase containing ketoconazole as an internal standard at a final concentration of 0.5 μ M. The concentration of compound in each sample was determined using LC-MS-MS.

2.11.4 MS

LC-MS/MS analyses was carried out using an Agilent (Santa Clara, CA, USA) 6410 triple quadrupole mass spectrometer equipped with positive-ion electrospray and a Model 1200 HPLC. The solvent system consisted of a linear 3-min gradient from 30–100% ACN in 0.1% aqueous formic acid and an Agilent XTerra C₁₈ HPLC column (2.1 mm \times 100 mm; 3.5 μ m) at 28°C. The flow rate was 200 μ L/min. After collision-induced dissociation of the protonated molecule of compounds at m/z 256, SRM was used to record the transition from m/z 256 to the abundant product ion of m/z 118 with a dwell time of 250 ms/ion. The SRM transition of m/z 531 to 489 was monitored for the internal standard ketoconazole. Propranolol and sucrose were measured using LC-MS as described by Li et al. [87].

2.12 Animal studies

Animal studies were performed to investigate oral absorption and dose-tolerance of compound 5 (protocol 101008929). Following oral administration (40 mg/kg body weight) for 3 days, tissues and serum were examined for parent compound and metabolites. Animals were sacrificed 24 h after the final dose. Liver, mammary, gland, perirenal fat, and serum were collected and stored at –80°C until analyzed. Serum and tissue homogenates were analyzed via LC-MS-MS. Analyses were carried out using a Shimadzu LC-20AD Prominence HPLC system interfaced with an Applied Biosystems API 4000 triple quadrupole mass spectrometer. A Waters XTerra MS C₁₈ column (2.1 mm \times 100 mm, 3.5 μ m) was used for HPLC separation. Positive-ion electrospray was used for ionization, and product ion mass spectra were recorded using collision-induced dissociation and SRM of the transition of m/z 333 to 298, m/z 353 to 166, and m/z 483 to 130 (300 ms per transition) for measurement of compounds and internal standard [88, 89].

3 Results

A total of 92 resveratrol derivatives were synthesized and tested. Biological responses and corresponding structure–function relationships of resveratrol and derivatives that functioned in a pleiotropic manner are summarized in Table 1. In a nitrite assay, the fundamental *trans*-stilbene structure (compound 75) showed 28.2% inhibition of nitric oxide (NO) production at a test concentration of 34 μ M. Resveratrol (1) mediated moderate activity with an IC₅₀ of 28.9 μ M. Incorporation of a carboxyl group, nitro group, or carbonyl at 4'-position in ring B did not improve inhibitory activity. However, addition of an amino group in the *para*-position (2) showed the most potent activity with an IC₅₀ of 3.7 μ M. Changing the location of the amino group to the *ortho*- or *meta*- position yielded IC₅₀ values of 7.2 and 10.2 μ M, respectively (17 and 3). None of the derivatives tested showed selective inhibition of NO production.

Resveratrol has relatively weak activity as an inhibitor of aromatase (IC₅₀ = 25 μ M), but a number of resveratrol analogs displayed much greater inhibition (Table 1). Of the 92 compounds tested, 32 were at least moderately active against aromatase. Of these, eight had IC₅₀ values less than 1 μ M, and two imidazole analogs inhibited aromatase activity by 50% at concentrations less than 100 nM (8, 20). These analogs are obviously superior inhibitors relative to the parent molecule of resveratrol. In addition, they are more potent than the commercially available aromatase inhibitor, aminoglutethimide (IC₅₀ = 0.2 μ M), although less active than some other inhibitors such as anastrozole and letrozole. Additional inhibitors are shown in Table 2.

Based on a luciferase expression assay, resveratrol (1) is a moderate inhibitor of NF κ B activity (IC₅₀ = 2.1 μ M). This

result encouraged further exploration of different derivatives as possible inhibitors of NF κ B. Among the 92 derivatives tested, 74 demonstrated weak to good inhibitory activity against NF κ B with IC₅₀ values in the 0.2–20 μ M range. Within this group, 11 compounds were more potent than resveratrol, with IC₅₀ values less than 2 μ M. Compound 2, with a *para*-amino group in the *trans*-stilbene backbone structure, showed greater inhibition of NF κ B than resveratrol. Some improvement was observed when an amino group in ring B was moved from the *para*- to the *meta*-position (3). Nevertheless, the majority of derivatives, with IC₅₀ values in the low micromolar range, have an amino group in the *para*-position (4, 6, 7, 12, 13). Addition of hydroxyl, methoxy, acetoxy, or methylenedioxy groups did not significantly change potency against NF κ B. The most active NF κ B inhibitor in the series was compound 16, which contains four nitro groups. The compounds listed in Table 3 demonstrated stronger inhibitory activity than the positive controls: TPCK (IC₅₀ = 5 μ M) and BAY-11-7082 (IC₅₀ = 2 μ M). Notably, these compounds mediated the inhibitory response with specificity.

Resveratrol induced QR1 activity with an IR of 2.4 and a CD of 21 μ M (Table 1). It appears that substitution of larger groups (acetyl, methoxy) in place of the hydroxy substituents results in increased induction. This can be seen in 12 (IR 2.8). It is also apparent that a 5,6-methoxy substitution on ring A increases induction, e.g. 17, (IR 3.7, CD 2.1). An amino group on ring B yields a more active compound than a nitro group, and the 2-amino group is optimal. Of the derivatives tested, specific induction of QR1 without affecting other targets was not observed.

Induction of QR1 is considered a biomarker for cancer prevention with a main function to inactivate harmful xenobiotic compounds [90]. On the other hand, with a distinctly different function, the enzyme QR2 is one of the highest affinity targets of resveratrol that has been identified to date [91]. To expedite the search for new inhibitors of QR-2, we developed two different high-throughput screening assays, one based on enzymatic activity and the other based on ultrafiltration LC-MS [92]. Compounds 5, 6, 11, 18, and 19 demonstrated dose-dependent inhibition of QR2 (Table 1) as well as other activities. As shown in Table 4, three compounds were identified with high QR2 inhibitory specificity.

ODC is a key enzyme in synthesis of polyamines. It is activated in different types of tumors and discovery of highly specific inhibitors of ODC is a promising approach in the field of cancer chemoprevention. Of the 92 derivatives tested, 36 compounds were found to actively inhibit the induction of ODC. They can be divided into two groups: those with IC₅₀ values in the range 10–20 μ M (eight compounds) and those in the range of 0.7–10 μ M (15 compounds). The most active derivative was resveratrol 4'-sulfate (IC₅₀ = 0.7 μ M, 14); another six inhibitors with high potency and specificity toward ODC are shown in Table 5.

As shown in Table 1, resveratrol selectively interacted with ER- α , as did compound 7. Other derivatives mediating pleiotropic activities did not generally show selectivity for estrogen type. However, some of the derivatives devoid of other activities did interact with ERs (Table 6). Among these, compounds 46 and 47 were ER- β selective, whereas other compounds bound to both ER- α and ER- β . The potent botanical estrogen 8-prenylnaringenin was included as a positive control, and it bound tightly to both ER- α and ER- β .

In the DPPH assay, compound 9, which has three –NH₂ groups, showed the most active scavenging activity with an IC₅₀ of 42.2 μ M. Substituting an amino group with a methoxy group (10, 11) produced good scavenging activity, although the resulting compounds are less potent than 9. Resveratrol had an IC₅₀ value of 78 μ M in the DPPH assay.

As demonstrated previously, resveratrol inhibits COX-1 and -2, and this is considered a target of physiological relevance. Of the derivatives tested, several were found to inhibit these enzymes with potency roughly comparable to the parent compound. None of the derivatives was found to selectively inhibit COX-2. However, as summarized in Table 7, five derivatives were found to inhibit COX-1 without affecting the other targets described herein.

All compounds were tested for anti-proliferation effects using three different cancer cell lines: MCF7, PC-3, and HL-60 (data not shown). Compound 10 showed the most potent activity in all three cancer cell lines: in MCF7 cells, the IC₅₀ was 0.02 μ M, and with PC-3 and HL-60 cells IC₅₀ values were 2 and 0.5 μ M, respectively. The second most active compound was 6, which has methoxy groups at the 3, 4, and 5 positions, and an amino group at the 4' position. However, the cytotoxic effect is decreased when one –OCH₃ is removed (5), the double bond between the two rings in the stilbene backbone structure is reduced (15), or the –OCH₃ position is changed (11).

3.1 Preliminary animal studies

Substituted stilbene 5 was found to possess characteristics attributable to a chemopreventive agent via different pathways (Table 1). It inhibits ODC induction (IC₅₀ of 1.3 μ M), modestly inhibits NF κ B (IC₅₀ of 5.8 μ M), induces QR1 by 1.9-fold, and inhibits aromatase with IC₅₀ value of 0.31 μ M. Compound 5 has been synthesized on a multi-gram scale [8]. To determine the feasibility of chemopreventive studies with animals, large-scale synthesis has been completed [8], and preliminary absorption and metabolism studies were performed. As shown in Table 7, the apparent permeability coefficients (P_{app}) of sucrose across the Caco-2 cell monolayers was $\sim 0.1 \times 10^{-6}$ cm/s, and the P_{app} of propranolol was $> 30 \times 10^{-6}$ cm/s, indicating that the Caco-2 cell monolayers utilized in this study possessed suitable monolayer integrity and function. The P_{app} values of compound 5 in the AP to BL direction ranged from 8.6 to 12.6×10^{-6} cm/s

Table 1. Resveratrol derivatives with pleiotropic mechanisms of action^{a)}

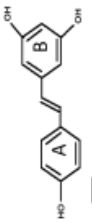
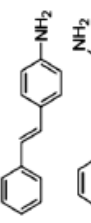
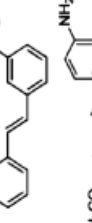
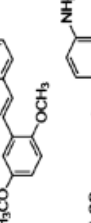
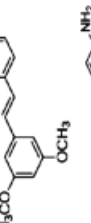
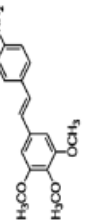
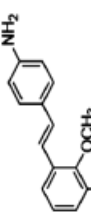
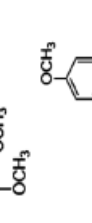
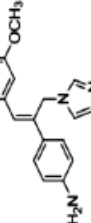
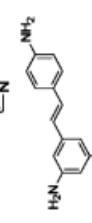
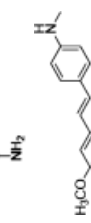
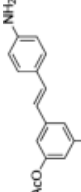
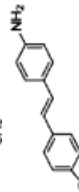
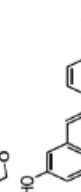
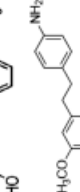
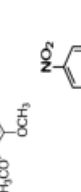
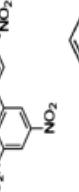
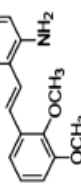
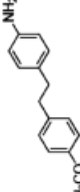
Compounds tested	NO ^{b)} IC ₅₀	Atom IC ₅₀	NFκB IC ₅₀	QR1 ^{c)} IR	QR1 ^{d)} CD	QR2 IC ₅₀	ODC IC ₅₀	DPPH ^{e)} IC ₅₀	ERα	ERβ	COX-1 ^{f)} IC ₅₀	COX-2 ^{f)} IC ₅₀
1 	28.9±1.0	25±2.6	2.1±0.15	2.4±0.29	21±0.46	6.9±0.4	8.8±1.18	78.0±1.1	5.32	1.23	0.35±0.1	2.3±0.28
2 	3.7±0.9	22±0.54	0.9±0.23	2.6±0.17	6.5±0.24	NT ^{g)}	18.1±2.1	147.9±0.3	NA ^{h)}	NA	2.2±0.34	2.5±0.6
3 	10.2±1.4	4.22±0.37	0.3±0.1	NA	NA	NT	6.4±0.8	NA	NA	NA	2.3±0.22	NA
4 	NA	0.31±0.09	0.3±0.15	NA	NA	9.0±0.5	5.2±1.4	258.0±1.0	2.82	2.06	0.61±0.22	2.3±0.32
5 	33.9±3.1	0.31±0.12	5.8±1.6	1.9±0.15	NA	1.7±0.06	1.3±0.4	227.7±0.5	1.65	1.33	0.76±0.14	12.4±5.1
6 	4.6±0.5	0.9±0.17	0.9±0.3	NA	NA	0.27±0.01	13.1±2.6	101.3±0.9	NA	NA	1.4±0.11	NA
7 	8.6±1.2	0.98±0.24	4.7±1.1	NA	NA	NA	NA	171.0±0.4	3.65	1.5	7.1±2.3	NA
8 	8.3±1.3	0.04±0.01	15.9±3.1	NA	NA	18.3±1.6	NA	NA	NA	NA	1.5±0.28	NA
9 	NA	8.53±0.62	6.5±0.9	NA	NA	34	1.3±0.16	42.2±0.3	1.23	3.21	4.1±2.1	NA
10 	NA	NA	11.6±1.9	NA	NA	NT	NA	135.8±0.6	NA	NA	3.8±1.5	NA
11 	14.7±2.7	2.42±0.37	17.3±3.1	NA	NA	1.5±0.07	NA	50.5±0.2	3.5	5.25	2.3±0.28	NA

Table 1. Continued

Compounds tested	NO ^{b)} IC ₅₀	Arom IC ₅₀	NF-κB IC ₅₀	QR1 ^{c)} IR	QR1 ^{d)} CD	QR2 IC ₅₀	ODC IC ₅₀	DPPH ^{e)} IC ₅₀	ER α	ER β	COX-1 ^{f)} IC ₅₀	COX-2 ^{f)} IC ₅₀
	31.2 ± 2.0	2.83 ± 0.25	0.5 ± 0.2	2.8 ± 0.16	5.6 ± 0.26	14.9	12.4 ± 1.9	NA	NA	NA	6.9 ± 3.4	NA
	16.6 ± 3.8	8.49 ± 0.48	0.3 ± 0.15	NA	NA	> 50	NA	87.9 ± 0.4	NA	NA	0.97 ± 0.21	NA
	NA	NA	18.2 ± 3.0	NA	NA	NA	0.7 ± 0.12	NA	NA	NA	0.46 ± 0.11	NA
	32.2 ± 0.4	2.80 ± 0.19	4.7 ± 1.1	NA	NA	33.8	4.2 ± 0.9	NA	NA	NA	5.0 ± 1.2	NA
	11.7 ± 1.9	4.30 ± 0.47	0.2 ± 0.11	2.3 ± 0.12	45.8 ± 0.87	NT	2.1 ± 1.3	NA	NA	NA	NA	NA
	7.2 ± 0.6	NA	6.1 ± 1.9	3.7 ± 0.16	2.1 ± 0.3	NT	12.3 ± 2.4	80.7 ± 0.3	NA	NA	NA	NA
	NA	1.8 ± 0.22	4.9 ± 0.72	NA	NA	2.5 ± 0.2	0.9 ± 0.3	88.6 ± 12	2.7	1.98	5.3 ± 1.4	NA
	NA	4.41 ± 1.23	0.7 ± 0.32	NA	NA	1.4 ± 0.08	NA	54.4 ± 8.2	2.17	2.91	1.1 ± 0.05	NA

a) Compounds were classified as pleiotropic if activity was demonstrated in three or more of the assays; all IC₅₀ values are in μ M.

b) 100% is with LPS as activator.

c) Induction ratio for treated versus control cells.

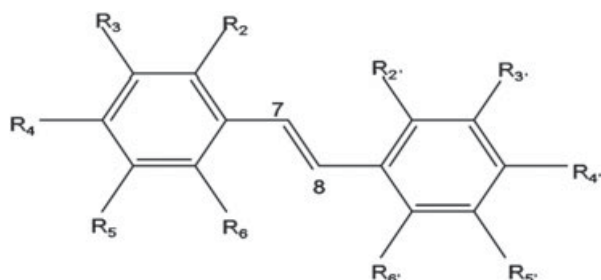
d) Concentration to double the expression of QR1.

e) Sample concentration that caused 50% inhibition of DPPH, 100% activity is with scavenging DPPH-free radical.

f) Concentration to inhibit COX-1 or -2 activity by 50%.

g) NT, not tested.

h) NA, not active, see Fig. 1 for activity criteria.



54 $R_3, R_6 = \text{OCH}_3$, $R_4 = \text{NO}_2$	68 $R_3, R_5, R_4 = \text{NO}_2$	81 $R_5 = \text{NH}_2$, 7-8 = CO-NH
55 $R_3, R_6 = \text{OCH}_3$, $R_6 = \text{NO}_2$	69 $R_3, R_5 = \text{OAc}$, $R_3 = \text{CO}_2\text{H}$	82 $R_4, R_5 = \text{OCH}_3$, $R_4 = \text{OAc}$
56 $R_3, R_5 = \text{NO}_2$, $R_4 = \text{OAc}$	70 $R_4 = \text{CO}_2\text{H}$	83 $R_4, R_5 = \text{OCH}_2\text{O}$, $R_4 = \text{NO}_2$
57 $R_4, R_5 = \text{OCH}_3$, $R_6 = \text{NH}_2$	71 $R_3 = \text{CO}_2\text{H}$, $R_4 = \text{OH}$	84 $R_5 = \text{NO}_2$, $R_4 = \text{NO}_2$
58 7-8 = CO-NH, $R_5 = \text{NH}_2$	72 $R_4 = \text{NO}_2$	85 $R_4, R_5 = \text{OCH}_3$, $R_4 = \text{F}$
59 $R_4, R_5 = \text{OCH}_3$, $R_5 = \text{NO}_2$	73 $R_3 = \text{OAc}$, $R_4 = \text{CO}_2\text{H}$	86 $R_3, R_5 = \text{OCH}_3$, $R_4 = \text{OAc}$
60 $R_4, R_6 = \text{OCH}_3$, $R_4 = \text{NO}_2$	74 $R_3 = \text{NO}_2$	87 $R_3, R_5 = \text{OCH}_3$, $R_4 = \text{CH}_2\text{NH}_2$
61 $R_4, R_6 = \text{OCH}_3$, $R_5 = \text{NO}_2$	75 All $R_s = \text{H}$	88 $R_3, R_5 = \text{OCH}_3$, $R_4 = \text{CN}$
62 $R_3, R_5 = \text{OCH}_3$, $R_4 = \text{CH}_2\text{NH}_2$	76 $R_3, R_5 = \text{KSO}_4$, $R_4 = \text{OK}$	89 $R_3, R_5 = \text{OCH}_3$, $R_5 = \text{NO}_2$
63 $R_3, R_5 = \text{OAc}$, $R_4 = \text{NO}_2$	77 $R_3, R_5, R_4 = \text{KSO}_4$	90 $R_5, R_6 = \text{OCH}_3$, $R_6 = \text{NO}_2$
64 $R_3, R_5 = \text{OCH}_3$, $R_4 = \text{Cl}$	78 $R_4 = \text{NO}_2$, 7-8 = CO-NH	91 $R_5, R_5 = \text{OH}$, $R_4 = \text{NO}_2$
65 $R_3, R_5 = \text{OCH}_3$, $R_4 = \text{F}$	79 $R_5 = \text{NO}_2$, 7-8 = CO-NH	92 $R_3, R_4, R_5 = \text{OCH}_3$, $R_4 = \text{CH}_2\text{NH}_2$
66 $R_4, R_5 = \text{OCH}_3$, $R_4 = \text{CN}$	80 $R_3, R_4 = \text{OCH}_3$, $R_6 = \text{NO}_2$	93 $R_4, R_5 = \text{OCH}_3$, $R_6 = \text{NO}_2$
67 $R_3, R_5 = \text{NO}_2$		

Figure 1. Compounds devoid of significant biological activity in the assays tested. (A) Each of the compounds shown in the figure were tested and found to be without significant activity by the following criteria ($\text{IC}_{50} > 1 \mu\text{M}$): NO (35); Aromatase (25); NF κ B (20); QR1 ($\text{CD} > 50$); QR2 (50); ODC (20); DPPH (340); COX-1 and -2 (68).

depending on the initial concentration. Since the permeability of **5** decreased as its initial concentration increased, participation of a saturable transporter was indicated. In the BL direction, the P_{app} values of **5** were 25–42% higher, which indicated that **5** is the substrate for an efflux protein. Treatment of the Caco-2 cells with the p-glycoprotein inhibitor verapamil enhanced the rate at which **5** crossed the Caco-2 monolayer in the AP to BL direction (Table 8). Therefore, **5** appears to be a substrate for p-glycoprotein.

Following oral administration to rats, the concentrations of **5** in plasma and tissue samples were determined using LC-MS-MS (Table 9). The level of **5** in serum was high ($3.21 \pm 0.12 \mu\text{g/mL}$) during the first day. After 3 days, this was reduced to $0.64 \pm 0.034 \mu\text{g/mL}$. Comparable concentrations were observed in mammary tissue and perirenal fat; the highest levels were in liver. These data indicate that high

serum and tissue levels can be achieved by oral administration, but dosing regimens should be further evaluated. In vitro studies with plasma proteins indicated $99.4 \pm 0.11\%$ (mean \pm SD; $n = 3$) of compound **5** was bound.

4 Discussion

As described herein, 92 resveratrol derivatives were synthesized and biological activity was assessed with various chemoprevention targets. As with the parent molecule, resveratrol, several of the derivatives mediated pleiotropic effects (Table 1). For example, compound **2**, with a *para*-amino group in *trans*-stilbene backbone structure, affected several targets, but inhibition of NO production was particularly notable. On the other hand, when methoxylated to yield compound **5**, pleiotropic responses were still observed,

Table 2. Selective inhibition of aromatase mediated by resveratrol derivatives

	Compounds tested	IC ₅₀ (μM) ^{a)}
20 ^{b)}		0.06 ± 0.01
21		0.68 ± 0.07
22		5.32 ± 1.12
23		0.2 ± 0.06
24		3.91 ± 0.22
25		1.84 ± 0.3
26		3.2 ± 0.29
27 ^{c)}		2.8 ± 0.14

a) Sample concentration that caused 50% inhibition of aromatase; Naringenin (IC₅₀ = 0.23 μM) was used as a positive control.

b) Compound 20 inhibited NFκB with and IC₅₀ of 13.1 ± 1.4 μM.

c) Compound 27 also inhibited COX-1 and -2 with IC₅₀ values of 5.3 ± 0.95 and 25.4 ± 6.6 μM, respectively.

but inhibition of aromatase and QR2 were especially enhanced. It is notable that resveratrol interacts with QR2 with a K_d value of 40 nM [91] and mediates inhibitory activity with an IC₅₀ value of 6.9 μM (Table 1). Compound 5 demonstrates greater inhibitory potential, and this is further enhanced by placement of an additional methoxy-group, as illustrated by compound 6. These compounds are calculated by molecular modeling to bind to the enzyme in a similar orientation to that of resveratrol, but interaction occurs with the side chain of Thr71, whereas resveratrol binds to this residue indirectly through a water molecule [8]. It may be

Table 3. Selective inhibition of NFκB mediated by resveratrol derivatives

	Compounds tested	IC ₅₀ (μM) ^{a)}
28 ^{b)}		0.2 ± 0.09
29		1.1 ± 0.23
30 ^{b)}		2.5 ± 0.16
31 ^{b)}		1.0 ± 0.19
32		0.7 ± 0.11
33 ^{b)}		0.5 ± 15

a) Sample concentration that caused 50% inhibition of TNF-induced NFκB activity treated with solvent (DMSO) only. TPCK and BAY-11 were used as positive controls (IC₅₀ = 3.8 and 2.0 μM, respectively).

b) Compounds 28, 30, 31, and 33 inhibited COX-1 with IC₅₀ values of 0.87 ± 0.04, 0.68 ± 0.2, 2.8 ± 0.67, and 2.0 ± 0.57 μM, respectively. Compound 31 inhibited COX-2 with and IC₅₀ value of 7.5 ± 1.6 μM.

suggested that these new analogs bind to QR2 with a K_d value in the 1–10 nM range.

Compound 5 was subjected to preliminary absorption and metabolism studies. High Caco-2 cell permeability suggests that intestinal absorption would be rapid and complete following oral administration. The in vivo data obtained using oral administration of 5 to rats are consistent with these in vitro Caco-2 cell monolayer permeability studies. Good absorption and stability were observed, unlike the parent molecule, which is known to be rapidly and extensively metabolized. In addition, a high level of binding with human plasma proteins was noted. Since only free drug would be available for metabolism or excretion, binding of this type might enhance the in vivo half-life, which would be beneficial for chemoprevention agents. However, strong binding to plasma proteins might also increase the probability of drug–drug interactions due to competition between drugs for plasma protein-binding sites.

In addition to compounds 5 and 6, several derivatives were found to inhibit aromatase activity in the sub-micro-

Table 4. Selective inhibition of QR2 mediated by resveratrol derivatives^{a)}

	Compounds tested	IC ₅₀ (μM)
34		4.1 ± 0.6
35 ^{b)}		2.1 ± 0.2
36		3.3 ± 0.5

a) Human QR2 was purified from *Escherichia coli* BL21. Frozen QR2, previously stored at –80°C, was thawed on ice and then adjusted to the 4 mg/mL concentration for IC₅₀ determination with different compounds.

b) Compound **35** inhibited COX-1 with and IC₅₀ value of 2.0 ± 1.4 μM.

Table 5. Selective inhibition of TPA-induced ODC activity by resveratrol derivatives

	Compounds tested	IC ₅₀ (μM) ^{a)}
37 ^{b)}		1.2 ± 0.22
38		1.0 ± 0.16
39		0.9 ± 0.24
40 ^{b)}		0.9 ± 0.15
41		0.8 ± 0.14
42 ^{b)}		2.1 ± 0.38

a) Concentration that caused 50% TPA-induced ODC activity. Apigenin (IC₅₀ = 6.0 μM), menadione (IC₅₀ = 8.3 μM), and deguelin (IC₅₀ = 0.1 μM) were used as positive controls.

b) Compounds **37**, **40**, and **42** inhibited COX-1 with IC₅₀ values of 1.19 ± 0.18, 1.92 ± 1.3, and 1.54 ± 0.64 μM, respectively. Compound **37** inhibited COX-2 with and IC₅₀ value of 1.41 ± 0.51 μM.

Table 6. Selective binding of resveratrol derivatives to human estrogen receptors^{a)}

	Compounds tested	ERα signal enrichment	ERβ signal enrichment
43 ^{b)}		3.99	4.87
44		2.57	ND ^{c)}
45 ^{b)}		2.17	2.91
46 ^{b)}		1.87	3.53
47 ^{b)}		1.76	4.62
48 ^{b)}		1.67	2.27

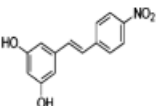
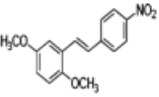
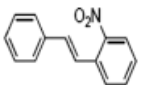
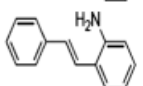
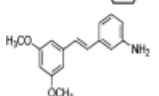
a) 8-Prenylnaringenin yielded values of 21.07 and 15.54 for ERα and ERβ, respectively.

b) Compounds **43**, **45**, **46**, **47**, and **48** inhibited COX-1 with IC₅₀ values of 0.8 ± 0.01, 2.8 ± 0.53, 0.9 ± 0.42, 1.2 ± 0.37, and 3.2 ± 0.75 μM, respectively. Compounds **43**, **46**, and **48** inhibited COX-2 with and IC₅₀ values of 1.7 ± 0.29, 6.4 ± 1.6, and 2.7 ± 0.73 μM, respectively.

molar range, e.g. **4** and **8**. The most promising inhibitors of aromatase, however, are shown in Table 2, with **21** and **23** mediating a selective response in the nanomolar concentration range. Analysis of structure–activity relationships revealed that the type and position of groups on ring B were more important for aromatase inhibitory activity than those on ring A. An amino group on ring B yielded more potent inhibitors than a nitro, halogen, hydroxyl, nitrile, aceto, or aminomethyl group. An amino group in the *para*-position resulted in greater inhibitory potency than the same group in an *ortho*- or *meta*-position. Although changing groups on ring A had less impact on enzyme activity, some conclusions could be drawn from the series regarding these groups. Methoxy groups on ring A yielded more inhibitory activity than hydroxyl or acetyl groups. A 3,5-arrangement of methoxy groups was found to be optimal. Thus, compound **5** [(*E*)-4-(3,5-dimethoxystyryl)aniline] was one of the most active compounds of the series,

with an IC_{50} of 0.31 μ M. Addition of an imidazole group increased inhibitory potency against aromatase significantly, and the most active compound in the series demonstrated an IC_{50} value of 36 nM (8). Molecular modeling indicated

Table 7. Selective inhibition of COX-1 mediated by resveratrol derivatives

	Compounds tested	IC_{50} (μ M) ^{a)}
49		0.46 ± 0.05
50		18.7 ± 1.9
51		14.9 ± 0.43
52		0.8 ± 0.15
53 ^{b)}		2.9 ± 0.82

a) Sample concentration that caused 50% inhibition of COX-1. Resveratrol was used as a positive control (Table 1).

b) COX-2 IC_{50} = 19.5 ± 1.4 μ M.

that one of the imidazole nitrogens coordinates with the iron atom of the aromatase heme, which plays a critical role in catalysis [8].

As demonstrated previously, NF κ B inhibition requires the planar conformation of resveratrol, preferring large, hydrophobic rings and a catechol at the R_3 position [80]. Table 1 illustrates active inhibitors of NF κ B with pleiotropic effects. Interestingly, bond reduction between rings negatively impacted sensitivity towards iNOS, aromatase, NF κ B, and growth inhibition with MCF7 and HL-60 cells (compare 6 and 15). In general, chemical modification does not greatly enhance NF κ B inhibitory potential (Table 1, refs. [80, 82]). However, as summarized in Table 3, six derivatives were identified that selectively inhibited NF κ B with IC_{50} values lower than that observed with resveratrol.

Resveratrol is a modest inducer of QR1. Of the derivatives tested, some were found to be approximately equipotent, but no selective inducers were found. Overall, compound 17 demonstrated the greatest promise. In contrast to the NADP-dependent QR1, which is up-regulated by cancer chemopreventive agents via the antioxidant-responsive element, the inhibition of QR2 by resveratrol or derivatives might protect cells from harmful metabolically activated compounds that can promote DNA damage and therefore cancer [12]. Three selective inhibitors were identified (Table 4) that should be of interest in the future work.

The strongest inhibitor of ODC induction was the natural metabolite resveratrol-4'-sulfate (14). We showed earlier that this metabolite inhibited NF κ B induction, as well as COX-1

Table 8. Apparent permeability coefficients of compound 5 across Caco-2 cell monolayers

Test compound (initial conc., μ M)	Apparent permeability coefficients (P_{app}) ^{a)} $P_{app} \times 10^{-6}$ cm/s		
	P_{app} (AP \rightarrow BL)	P_{app} (BL \rightarrow AP)	Ratio
5 (10)	$12.6 \pm 0.9^a)$	15.8 ± 1.4	0.79
5 (25)	10.5 ± 2.4	14.1 ± 0.2	0.74
5 (50)	8.6 ± 1.0	12.2 ± 0.5	0.70
5 (10)+Verapamil (50)	24.9 ± 0.3	14.1 ± 0.2	1.8
5 (25)+Verapamil (50)	13.8 ± 0.2	15.1 ± 2.9	0.91
5 (50)+Verapamil (50)	8.6 ± 0.8	n.d. ^{b)}	
Sucrose (50)	0.14 ± 0.04		
Propranolol (10)	32.1 ± 1.2		

a) Mean \pm SD (N = 3).

b) Since the rate at which 50 μ M compound 2 crossed the Caco-2 cell monolayer in the basolateral to apical direction was non-linear in the presence of verapamil, the P_{app} value could not be calculated.

Table 9. In vivo disposition of compound 5 in the rat

Compound 5 40 mg/kg	Serum 1-day (μ g/mL) N = 9	Serum 3-day (μ g/mL) N = 9	Liver (μ g/g) N = 9	Mammary gland (μ g/g) Pooled, N = 3	Fat (μ g/g) Pooled, N = 3
Rat 1	2.99 ± 0.12	1.04 ± 0.005	4.68 ± 0.57	–	–
Rat 2	2.90 ± 0.09	0.67 ± 0.005	9.88 ± 0.057	–	–
Rat 3	4.00 ± 0.06	0.21 ± 0.001	5.61 ± 0.91	–	–
Total	3.21 ± 0.12	0.64 ± 0.034	6.28 ± 2.36	0.51 ± 0.01	0.69 ± 0.04

and COX-2 activities [82]. Rapid metabolism of resveratrol during oral administration leads to production sulfates and glucuronides [93]. The ability of sulfate metabolite to inhibit ODC further supports the potential importance of resveratrol metabolites, although other derivatives are selectively active in this process (Table 5).

Estrogen regulates growth, differentiation, and functioning of many target tissues. Resveratrol, being structurally similar to the synthetic estrogenic compound diethylstilbestrol, has been demonstrated to be a competitive inhibitor of binding to the ER in human breast cancer cells MCF7 [94]. The estrogenic activity of resveratrol as an agonist of ERs has been documented [95], although the relevance of such an effect is moot [7]. ERs α and β (ER1 and ER2) are members of the nuclear receptor family, which controls many processes in cells, including cancer development. Binding of agonistic or antagonistic molecules to these receptors is a well-established approach in the search for anticancer agents. As shown in Table 1, resveratrol selectively interacts with ER- α , as did compound 7. Other derivatives such as 46 and 47 were ER- β selective. It would be of value to study these types of interactions in greater detail by means of X-ray crystallographic analysis. These compounds should be of value in performing such studies.

With our experimental conditions, resveratrol inhibits COX-1 and COX-2, but greater activity was observed with COX-1 (Table 1). Similar to resveratrol, several derivatives active as COX inhibitors blocked both forms of the enzyme (2, 4, 5, 27, 31, 27, 43, 46, 48), but no COX-2 specific inhibitors were found. On the other hand, five compounds were identified as selective inhibitors of COX-1. The significance of this response remains to be explored. It may be noted, however, resveratrol mediates a significant anti-inflammatory response in a rat model [1], and, as noted above, greater activity is observed with COX-1.

The structure-activity relationships (SARs) that can be derived from the data set described herein are extensive. Some highlights are given in the text but certainly additional concepts can be gleaned from these results. Relative to resveratrol, it is clear that improvements in activity can be achieved. In some cases, as with resveratrol, pleiotropic activities can be observed with derivatives, albeit with greater potency. On the other hand, a total lack of activity can be generated, as shown in Fig. 1, which can teach us something about SARs. Perhaps of greatest interest are chemical derivatives that demonstrate much greater specificity with much greater potency. In many cases, these novel compounds demonstrate activity even greater than the positive control compounds used for the respective assays, suggesting further exploration as therapeutic or preventative agents is reasonable.

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