

THE WORLD OF RESVERATROL

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INTRODUCTION

Since the discovery of *trans*-resveratrol (3,5,4'-trihydroxystilbene) as a constituent of wine by Siemann and Creasy, first reported in 1992 (1), the possibility that this compound, almost unique to red wine among constituents of the human diet, may in large measure account for the putative health benefits of this beverage beyond its mere content of vulgar ethanol, excited the imagination of the scientific and medical communities, initiating a ferment of research and enquiry that continues to this day. Indeed, ripples of these activities from time to time flow into the pages of the lay press, so that resveratrol has become a molecule impacting the consciousness of many well-informed members of the lay public. In March, 1997 we published a major review incorporating 183 references forming the bulk of the world literature on resveratrol up to that time (2). Our *bottom line* was that the future of resveratrol did not look particularly promising given the reality that, despite its miraculous performances in the culture dish and the test tube, the intact

bodies of mice and men proved to be an inhospitable milieu robbing it of its presumed powers. To us, it seemed to be a compound *for whom the bell tolled*, but others did not share this gloomy prognosis. In fact, so much new work on this topic has been published in a mere two years-and-a-bit that a reappraisal of the situation deserves a welcome and is mandated by the present Symposium. Resveratrol exists as *trans* and *cis* isomers. Very little is known about the latter. When the nature of resveratrol is not specified, the reader should assume that the text refers to the *trans* isomer.

Before plunging into the biological effects of resveratrol, some background concerning its chemical nature, natural occurrence and biosynthesis would appear to be desirable. These themes were in fact extensively described in our earlier review (2), but a brief synopsis of these themes is not out of place and should prove helpful. The statements made will not be referenced since the relevant primary literature is already cited (2). The only further preliminary task is to draw attention to two other short reviews that describe its functions in plant biology (3) and human health (4), respectively.

OCCURRENCE AND FUNCTION OF RESVERATROL IN THE PLANT KINGDOM

More than 30 stilbenes and stilbene glycosides occur naturally among members of the plant kingdom classified as spermatophytes. The essential structural skeleton comprises two aromatic rings joined by a methylene bridge. Resveratrol is a pivotal molecule in plant biology with homologies extending into the realm of mammalian fatty acid metabolism. Its main significance lies in its role as the parent molecule of a family of polymers given the name viniferin. These compounds are able to inhibit fungal infection, a property which has earned their inclusion in the class of plant antibiotics known as phytoalexins. Until 1992, there was no interest in resveratrol from the perspective of mammalian biochemistry or clinical science, but in that year Siemann and Creasy reported the presence of *trans*-resveratrol in wine and drew attention to the fact that it was also a constituent of oriental folk medicines reputed to benefit persons afflicted by a wide range of disorders.

Resveratrol does not enjoy a wide distribution in the plant world, and has been reported in few fruits and vegetables employed for human consumption (Table 1). One of the richest sources is the weed *Polygonum cuspidatum*, root extracts of which have played an important role in Japanese and Chinese folk-medicine. Its occurrence has been documented in a number of trees: these include eucalyptus and spruce. Cotyledons of groundnuts (*Arachis hypogaea*) synthesise an array of phytoalexin stilbenes, including

resveratrol, concentrations of which are greatly increased in response to infection, wounding, and irradiation with ultraviolet (but not visible) light.

Most interest has centered upon resveratrol in grapevines (*Vitaceae*) because its function as a phytoalexin and its role as a marker of infection by various pathogens has been intensively investigated in this genus. The first reports describing the presence of resveratrol in grapevine tissues

Table 1. Natural Occurrence of Resveratrol Among Spermatophytes

Type	Species
Weeds	<i>Polygonum cuspidatum</i>
Trees	<i>Eucalyptus</i> Spruce <i>Bauhinia racemosa</i> Scottish pine (<i>pinosylvin</i>)
Plants	<i>Veratrum formosanum</i> <i>Veratrum grandiflorum</i>
Legumes	<i>Pterolobium hexapetallum</i> (non-edible)
Nuts	<i>Arachis hypogaea</i>
Vines	<i>Vitaceae</i> . Present in roots, Canes, leaves and berry skin

and its induction by fungal infection emphasized that ultraviolet irradiation, but not natural sunlight, could stimulate its synthesis as well as that of the viniferins. Whereas none of these phytoalexins are present in healthy vine leaves or berries, they are quite abundant in mature vine wood. Detailed investigations demonstrated a relationship between susceptibility to fungal infection (*Botrytis cinerea*), and the concentrations of stilbenes (resveratrol and viniferins) in the leaves and berries of different *Vitaceae*. Stilbene production in leaves and berries were positively correlated, and there was a negative correlation between stilbene production and susceptibility to *B. cinerea*. Without infection, only very low concentrations of both stilbenes could be detected in these parts of the vine. Resveratrol is not present in the berry flesh, but only in the skins, with very low concentrations found in the latter in the absence of induction. Resistant species produced five-times the maximum concentrations of resveratrol as susceptible species. The concentrations were greatest in the non-infected fruit close to necrotic lesions and appeared to help in limiting their spread. It appears that resveratrol production enables the vine to withstand *Botrytis* attack until climatic

conditions (mild and humid) favourable to the pathogen tip the balance against the host. The stimulus to resveratrol production in healthy berries at some distance from infected areas must take the form of a chemical signal regulating stilbene synthase activity, generated by the pathogen or by the infected berries.

BIOSYNTHESIS AND GENE TRANSFER

Resveratrol and related stilbenes are strongly implicated as an important mechanism in host defense against infection and injury among those members of the plant community that can conjure up its existence. Since the gene specifying its synthesis enjoys a very restricted distribution, it is not surprising that crops subject to endemic fungal attack from which it is constitutively absent have become the target of genetic engineering in an attempt to correct this natural deficiency.

The immediate precursors of resveratrol are *p*-coumaroyl CoA and malonyl CoA in a molar ratio of 1-to-3. The latter is derived by elongation of acetyl CoA units and the former from phenylalanine which, in plants, can be synthesised from sugars via the shikimate pathway. The condensation of *p*-coumaroyl CoA with three molecules of malonyl CoA is accomplished through the activity of the stilbene synthase, resveratrol synthase, in most species of the *Vitaceae* and four moles of CO₂ are released for each mole of resveratrol synthesised (Figure 1).

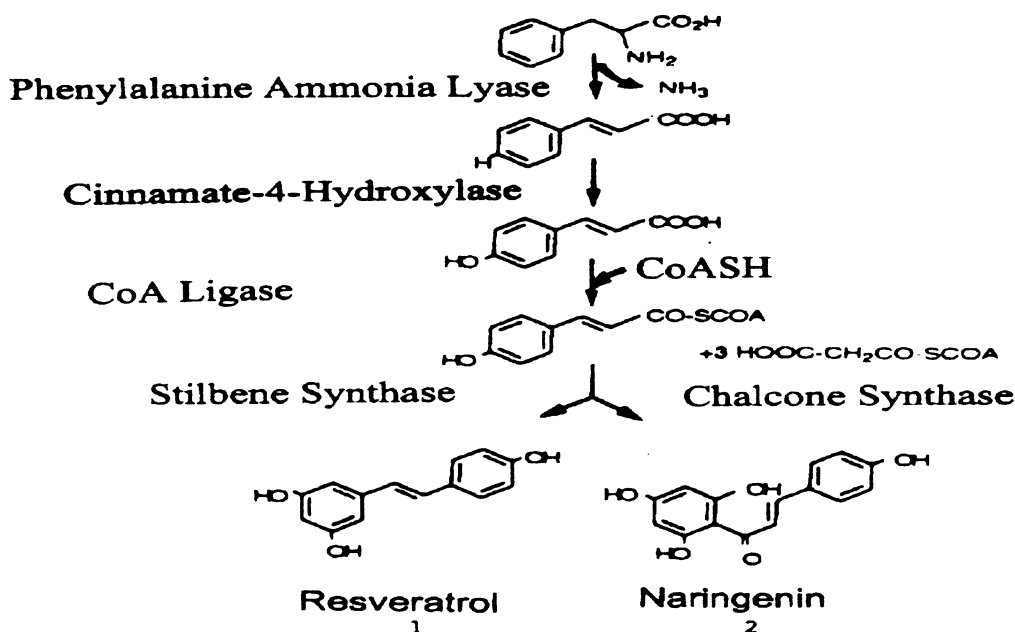


Figure 1. Biosynthetic pathway from phenylalanine to resveratrol.

The *Vitaceae* possess another enzyme, chalcone synthase, which catalyses a reaction involving one molecule of *p*-coumaroyl CoA and three of malonyl CoA, but in this instance only three molecules of CO₂ are generated; the other product is naringenin chalcone which, in a further series of reactions, gives rise to the flavonoid family.

Resveratrol synthase and chalcone synthase are different in this fundamental respect; the latter is expressed constitutively, such that its activity is substrate-driven and its products accumulate in proportion to the generation of precursor sugars during ripening and maturation of the berries, a process stimulated by UV light and possibly accounting for the ability of the latter to modulate its mRNA production. By contrast, the former is normally unexpressed and is inducible only by a range of provocations which include UV-irradiation, trauma and infection. Because of its potential to confer disease resistance upon plants incapable of performing its synthesis, resveratrol and stilbene synthase have been the targets of investigations to transfer the gene specifying its synthesis to disease-susceptible plants lacking this genetic information. The first step was the expression in *E. coli* of a full-length stilbene synthase cDNA prepared from grapevine mRNA yielding an enzymatically-active dimer exhibiting only stilbene synthase activity. Next, the gene from groundnut (*Arachis hypogaea*) that codes for stilbene synthase was transferred to tobacco plants (*Nicotiana tabacum*). Three years later, evidence was presented that the regenerated tobacco plants containing these genes were more resistant to *B. cinerea* infection than the wild type.

INFLAMMATION AND ATHEROSCLEROSIS

These disease processes involve mechanisms common to both, notably activation of polymorphonuclear leukocytes (PMN) with release of cytokines and synthesis of pro-inflammatory eicosanoids. Oxygen free radicals and immune responses also play important roles in their initiation and propagation. By contrast, lipid abnormalities predispose to atherosclerosis but not inflammation as such, while infectious agents are common precursors of inflammation. However, these distinctions are becoming increasingly blurred as our knowledge expands to reveal that in many respects atherosclerosis behaves as an inflammatory disease (5), and that micro-organisms may play a role in its etiology (6).

Resveratrol has been shown to modulate a number of metabolic and enzymatic pathways that are central to the inflammatory response, and has been reported to inhibit carrageenan-induced injury in the mouse (7). These authors attributed the observed protection to down-regulation of cyclooxygenase (COX) I, a constitutively expressed enzyme responsible for the biosynthesis of prostaglandins and thromboxanes. Subbaramaiah *et al* (8) subsequently described inhibition by resveratrol of the inducible enzyme

COX II that is increased following stimulation by mitogens such as phorbol ester. This inhibition operated at several levels: direct enzymatic activity, protein and mRNA synthesis, a cyclic AMP response element, protein kinase C, and AP-1-mediated gene expression. Their experiments were performed with human mammary epithelial cells and with resveratrol concentrations in the range 2.5-30 μM . Paradoxically, the same group of investigators were unable to detect resveratrol-induced changes in the COX I or COX II content of mouse skin cells stimulated by phorbol ester (9,10). However, the production of prostaglandin E_2 by an osteoblastic cell line was inhibited by resveratrol (11). It appears, therefore, that the alterations in COX gene expression by resveratrol are not identical in different experimental models of tissue damage, and that they may manifest tissue specificity dependent upon the response evoked by the compound on the expression of c-fos.

In actual fact, COX acts in conjunction with a peroxidase to comprise the Prostaglandin H Synthase (PGHS) multienzyme system. Employing a different *in vitro* model, Johnson and Maddipati found that resveratrol inhibits PGHS-1, but causes a 2-fold increase in the activity of PGHS-2 (12). Inhibition of the peroxidase activity of the former took place with an IC_{50} of 15 μM ; the peroxidase activity of the latter was inhibited with an IC_{50} of 200 μM . These data are inconsistent with a number of contemporaneous reports. Resveratrol appears to inhibit the COX activity of PGHS-2 purified from sheep seminal vesicles although with a potency that is only 25-30% of the inhibitory capacity manifested by the resveratrol polymer α -viniferin (13,14). It also inhibits a COX-like enzyme, tentatively identified as a COX-2, from the invertebrate *Ciona intestinalis* (15).

Formation of fibrous tissue to replace necrotic cells is a fundamental and irreversible part of the chronic inflammatory process. In the liver, stellate cells subserve this function. Kawada *et al* demonstrated inhibition by resveratrol of rat hepatic stellate cell proliferation using an *in vitro* model (16). Simultaneously, the following functions were decreased: activity of mitogen-activated protein (MAP) kinase; concentration of the cell cycle protein, cyclin D1; production of nitric oxide and of the pro-inflammatory cytokine tumor necrosis factor α (TNF- α). These effects would be expected to reduce both acute and chronic inflammatory reactions in the liver, but the resveratrol concentrations used were quite high (10-100 μM). A possible protective effect of resveratrol against immunological hepatocyte damage assessed by release of the enzyme alanine aminotransferase into the medium has recently been demonstrated in experiments with cultured hepatocytes (17).

The inhibition by resveratrol of COX and PGSH *in vitro* leads to marked reduction of eicosanoid production in affected cells. This was first demonstrated for rat peritoneal polymorphonuclear leukocytes (18) in which the cyclo-oxygenase pathway (evaluated by the synthesis of HHT and thromboxane B_2) was blocked (IC_{50} around 0.5-1 μM), as was the 5-

lipoygenase pathway (assessed by 5-HETE production, IC_{50} 2.72 μ M). It was subsequently shown that resveratrol inhibited the 5-lipoxygenase and 15-lipoxygenase pathways in washed neutrophils from healthy human subjects with IC_{50} concentrations of 22.4 μ M and 8.7 μ M, respectively (19), in line with the earlier observations of Kimura and colleagues (20) who reported that resveratrol prevented the formation of an array of 5-lipoxygenase products in human leukocytes (IC_{50} values 1.37-8.90 μ M).

The synthesis of eicosanoids by platelets is also blocked by resveratrol. A series of papers by Chinese investigators described its inhibition of thromboxane B_2 production from arachidonate in rabbit platelets (21-23). Similar inhibition of thromboxane B_2 synthesis in human platelets was reported, as well as a modest reduction in the activity of the platelet 12-lipoxygenase pathway leading to the production of pro-atherogenic hepoxillins (24).

Aggregation of platelets is a prelude to thrombus formation, the mechanism that precipitates coronary artery occlusion in the majority of patients who go on to develop acute myocardial infarction. This phenomenon is prevented by resveratrol in rabbit platelets (22,23) and in human platelets (24); in the latter, the IC_{50} is around 10 μ M with ADP or thrombin as agonist. Bertelli and colleagues (25,26) reported an IC_{50} for *trans*-resveratrol with human platelets of 15 nmol/l and a slightly lower value for *cis*-resveratrol when collagen was employed as agonist. These are orders of magnitude less than IC_{50} values for various biological effects reported by virtually all other investigators. Moreover, no information was provided about the nature of the *cis*-resveratrol whose synthesis has never been reported, apart from the name of the institution from which it was obtained. Confirmation of the antiplatelet aggregation activity of resveratrol has recently been provided (27).

Contact between circulatory blood cells, especially polymorphonuclear leukocytes and monocytes, and the vascular endothelium is a necessary prelude to the entry of these cells into the underlying intimal layers where the latter can undergo transformation into macrophages that then take up lipids, particularly oxidized LDL, to become the 'foam cells' characteristic of the early atherosclerotic lesion known as the 'fatty streak'. This contact is facilitated by an array of adhesion molecules whose expression is up-regulated by endothelial damage and cytokine signalling typically observed in atherosclerosis (28,29). Resveratrol, in concentrations ranging from 100 nM to 1 μ M blocked the expression of at least two of these adhesion molecules in $TNF\alpha$ -stimulated human umbilical vein endothelial cells (30), as shown in Figure 2. It also reduced the expression of the β_2 integrin MAC-1 on the surface of activated human polymorphonuclear leukocytes (31). Both of these functions could have important implications for the potential anti-atherosclerotic role of resveratrol.

The release of lysosomal enzymes from activated PMN leukocytes causes degranulation and contributes to inflammatory damage in adjacent

tissues. Resveratrol prevents this secretion in cells stimulated by the calcium ionophore A23187, but the IC_{50} has been reported to be around 0.1-1 mM by

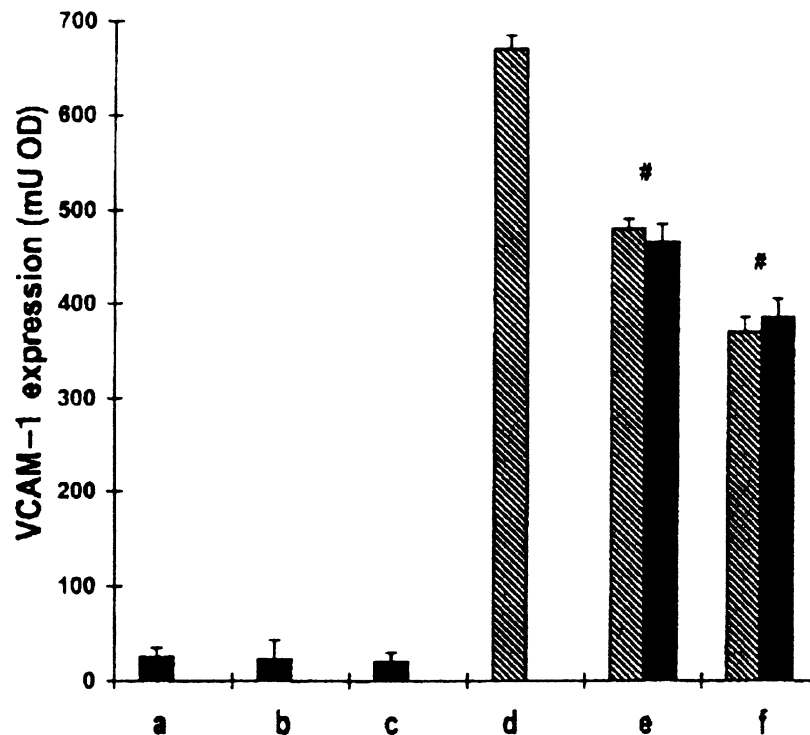


Figure 2: Inhibition by resveratrol of LPS-induced VCAM-1 expression of vascular endothelial cells. The letters represent the following conditions: a. unstimulated cells; b, same with 100 nM resveratrol; c. same with 1 μ M resveratrol; d, cells stimulated with LPS; e, same with 100 nM resveratrol; f, same with 1 μ M resveratrol; #, $P < 0.05$ (From Ref. 30, with permission).

one group (20) and around 30 μ M by other investigators (31). The liberation of β -hexosaminidase from cultured RBL-2H3 cells was also inhibited by resveratrol with an IC_{50} of 14 μ M (32).

An important inflammatory pathway involves activation of the transcription factor NF- κ B which promotes the synthesis of several cytokines, including TNF α , and nitric oxide (NO); the former can then lead to the release of pro-inflammatory tissue factor (TF). Resveratrol seems to disrupt this pathway, although there is some disagreement about how these effects are accomplished. Tsai *et al* (33) described inhibition of NO generation accompanied by down-regulation of NF- κ B in a macrophage cell line stimulated by lipopolysaccharide (LPS) at a resveratrol concentration of 30 μ M. Wadsworth and Koop (34) reported that, in concentrations in the range of 50-100 μ M, resveratrol did not inhibit LPS-induced activation of NF- κ B in the same cell line. It did reduce LPS-induced NO release but enhanced LPS-induced production of TNF α . Oxidized lipoproteins activate NF- κ B binding to the promoter region of target genes in PC12 cells, a rat

pheochromocytoma cell line, but resveratrol protects the cells against this activation and apoptotic cell death that follows (35). All three groups measured NF-KB activation by the same technique (electrophoretic mobility shift assay).

A further set of investigators found that resveratrol displayed a dose-dependent inhibition of TF expression in endothelial cells stimulated by a variety of agonists, including TNF α and LPS (36). They also showed that resveratrol inhibited LPS-induced expression of TNF α and IL-1 β in endothelial cells and monocytes. However, these phenomena could not be attributed to activation of transcription factors (including NF-KB) necessary for induction of the TF promoter in these cells. Resveratrol has also been reported to block the dioxin-induced increase of IL-1 β in an endometrial adenocarcinoma cell line (37). Further, production of phorbol ester-induced TGF- β 1 in mouse skin was inhibited by resveratrol (9,10), although these authors were unable to detect an increased TNF α content in these cells. To summarize, it does appear that resveratrol can attenuate the production of cytokines by vascular cells and peripheral blood cells, but the mechanisms involved remain to be elucidated, especially the issue of whether these effects are indirectly due to radical scavenging activity or whether they are associated with direct alteration of gene expression.

The possibility that resveratrol may modulate lipid metabolism was first proposed by Arichi *et al* (38) who provided resveratrol both orally and intraperitoneally to rats and mice fed a high cholesterol diet, and noted reduced deposition of cholesterol and triglyceride in the livers of these animals, as well as a diminished rate of hepatic triglyceride synthesis. Our group utilized the human hepatoma cell line Hep G2 to study the effects of resveratrol upon lipid and lipoprotein metabolism (19,39). The intracellular content of cholesteryl esters and the rate of secretion of both cholesteryl esters and triglycerides were reduced in a dose-dependent manner over concentrations of 1-50 μ M. Under these conditions the intracellular content and rates of secretion of apolipoprotein B (the main protein of VLDL and LDL) and of apolipoprotein AI (the main protein of HDL) were also reduced; since the former change would tend to prevent and the latter to augment atherosclerosis, these effects would seem to cancel each other out. When wines of high and low resveratrol content were administered to healthy humans for a period of 4 weeks, there was no major difference in the plasma lipid and apolipoprotein responses between the two experimental groups (40). An absence of change in serum lipoprotein patterns in the rat following the intraperitoneal injection of large doses of resveratrol has also been reported (41). On balance, it appears that resveratrol does not have a beneficial effect on circulating lipid or lipoprotein concentrations and that its anti-atherosclerotic properties *in vivo*, if any, are not attributable to such effects. Indeed a disturbing report published a few years ago described an *increase* in the area of aortic atherosclerosis visualized in resveratrol-fed hypercholesterolemic

rabbits compared with controls (42). The resveratrol was given in a dose of 0.6 mg/kg during the first 5 days and 1 mg/kg from days 6-60, but this amount (up to 3 mg) was stated to be dissolved in 0.05 ml of ethanol, vastly in excess of its solubility.

CELL GROWTH, PROLIFERATION, AND CANCER

When added to cultured Hep G2 cells in concentrations ranging from 1-50 μM , resveratrol did not alter the following functions over time periods up to 7 days: number of cells per plate; cell viability as gauged by trypan blue exclusion and lactate dehydrogenase efflux; incorporation of [^{14}C]-leucine into cell proteins. However, [^{14}C]-thymidine incorporation into DNA was stable for 3 days and showed a sharp increase at day 7, but only at 50 μM concentration (39). Two years later Jang *et al* (7) reported that resveratrol (1-25 μM) inhibited the initiation and promotion of hydrocarbon-induced skin cancer in the mouse as well as the progression of breast cancer in the same animal. A potent antimutagenic activity of resveratrol was also demonstrated (43). Subsequently, resveratrol has been shown to behave as an antiproliferative agent in estrogen-dependent as well as estrogen-independent human breast epithelial cells (44), in a human oral cancer cell line (45), in androgen-responsive and androgen-nonresponsive human prostate cancer cell lines (46), and in the Yoshida AH-130 ascites hepatoma inoculated into rats (47).

One mechanism responsible for this behaviour seems to be induction by resveratrol of apoptosis. This was first described in resveratrol-treated HL-60 cells, a human leukemia cell line, and to be mediated by a dose-dependent increase in intracellular caspases as well as CD-95L expression (48). Apoptosis of Yoshida AH-130 ascites tumour cells in response to resveratrol was demonstrated by flow cytometric analysis (47). By contrast, normal human lymphocytes were unaffected. In cells expressing wild-type p53, but not in p53-deficient cells, resveratrol suppression of tumor promoter-induced cell transformation is accompanied by apoptosis together with transactivation of p53 activity and expression of p53 protein (49). The dose responses for these apparently related phenomena manifest a similar pattern. Androgen-responsive human prostate cancer cells also undergo apoptosis in response to resveratrol (46). Paradoxically, resveratrol was reported to block apoptosis induced by oxidized lipoproteins in PC-12 cells, an outcome that is preceded by NF-KB activation and binding to DNA (35).

Arrest of cell division is an alternative mechanism for resveratrol-induced inhibition of cell growth, and may be accompanied by enhanced differentiation or similar phenotypic changes in growth-arrested cells. This was first demonstrated for HL-60 cells that, at a concentration of 30 μM , became arrested at S-phase concomitant with significant increase of cyclins A

and E and of cdc 2 in the inactive phosphorylated forms (44). However, rat hepatic stellate cells did not show any increase in these cyclins in response to resveratrol, although cyclin D1 content was reduced (16). Differentiation of the HL-60 cells towards a myelo-monocytic phenotype simultaneously occurred (50). These findings are consistent with an earlier report that resveratrol inhibits ribonucleotide reductase (IC_{50} around 4 μ M), the enzyme that provides proliferating cells with deoxyribonucleotides required for DNA synthesis during early S-phase (51). Indeed, its potency on a molar basis was orders of magnitude greater than that of hydroxyurea, another inhibitor of the same enzyme that has been used therapeutically as an anticancer and anti-HIV agent. Resveratrol also leads to an increase in the proportion of androgen-nonresponsive human prostate cancer cell lines in S-phase, but this does not occur with androgen-responsive cells (46). It will be recalled that the latter undergo apoptosis in response to resveratrol whereas the former do not. Resveratrol inhibits another important enzyme involved in DNA synthesis, DNA polymerase (52), and causes cleavage of DNA in the presence of Cu^{2+} ions (53). At variance with the above findings is a report that resveratrol in low concentrations (10^{-9} - 10^{-7} M) dose-dependently increased DNA synthesis, proliferation, and differentiation of osteoblastic MC 3T3-E1 cells (11). This action was blocked by the antiestrogenic compound tamoxifen.

In addition to its putative antimutagenic, pro-apoptotic, and DNA antisynthetic properties, resveratrol may target another biological system involved in carcinogenesis. Aryl hydrocarbons such as dioxin and dimethylbenzanthracene are taken up by a specific cytosolic receptor (AHR) in susceptible cells. The complex translocates to the nucleus where it dimerizes with another protein and initiates the transcription of a number of genes involved in carcinogenesis. The best characterized of these is the *CYP1A1* gene that encodes a cytochrome P_{450} -dependent microsomal enzyme; the *CYP1A1* gene product hydroxylates aryl hydrocarbons to genotoxic metabolites that bind DNA with consequent mutational events. Ciolino and colleagues reported that resveratrol in concentrations between 0.5 and 20 μ M inhibited the induction of CYP1A1 protein, mRNA and enzyme activity by the halogenated dioxin derivative TCDD in Hep G2 cells in a dose-dependent manner (54). It also prevented the TCDD-induced transformation of cytosolic AHR to its nuclear DNA-binding form and blocked its binding to promoter sequences that regulate CYP1A1 transcription. It did not modulate the binding of TCDD to cytosolic AHR.

These results were partially confirmed by Casper *et al* in a human breast cancer cell line, but with some differences (37). They found that resveratrol displaced labelled dioxin from AHR with an IC_{50} of 6 μ M. Neither nuclear translocation or DNA binding of AHR were altered by resveratrol, but its transcriptional activity for CYP1A1 was blocked. The most novel and exciting aspect of this report was the finding that resveratrol, when given in doses of 1 and 5 mg/kg to rats, inhibited the expression of CYP1A1 in lung

and kidney induced by benzpyrene and dimethylbenzanthracene. The likely tissue concentrations of resveratrol were three orders of magnitude less than those required for CYP1A1 inhibition in the *in vitro* experiments, suggesting that resveratrol may be converted *in vivo* to a metabolite one thousand-fold more active than the parent compound. It should be added that resveratrol in concentrations $<1 \mu\text{M}$ inhibited a range of cytochrome P₄₅₀-linked enzymes in hamster liver microsomes *in vitro* (55), but it also induced the mRNA for CYP1A1 in cultured Hela cells derived from a human cervical carcinoma (56). Finally, modulation of c-fos gene expression has been postulated to be an important target for the anticancer activity of resveratrol, at least in the mouse skin carcinogenesis model (9,10).

An important and practical conundrum surrounding the interaction of resveratrol with cancer cells is whether the former is able to suppress the production by the latter of tumor-specific cancer marker proteins. Hsieh and Wu (45) reported a decrease in the intracellular and secreted prostate-specific antigen (PSA) content of an androgen-sensitive prostate cancer cell line. Utilizing human breast cancer cell lines, we have been unable to demonstrate an effect of resveratrol upon the secretion of either PSA or carcinoembryonic antigen (CEA) in four different breast carcinoma cell lines. Nor could we demonstrate any changes in cancer-associated p53 gene expression even though the presence of genetic mutations in these cell lines was structurally determined by DNA analysis.

ANTIOXIDANT ACTIVITY

The ability of *trans*-resveratrol to function as an antioxidant was first demonstrated by Frankel *et al* (57). On a molar basis it was less effective than a number of flavonoids in preventing the copper-mediated oxidation of human LDL, but it was much more potent than α -tocopherol. Frankel *et al* (58) examined the relative contribution of individual wine phenolics to the inhibition of LDL oxidation, based upon their concentrations in the wines utilized, and concluded that resveratrol did not correlate with this activity. Later, Soleas *et al* (59) reported that resveratrol contributed significantly to the total antioxidant activity of wine as evaluated using the Randox *in vitro* assay.

Belguendouz *et al* carried out an extensive examination of the inhibition by resveratrol of porcine LDL oxidation in the presence of the free radical generator AAPH or copper ions (60). The slope of the propagation phase and the prolongation of the lag phase were much greater with the latter than with the former. Formation of thiobarbituric acid-reactive substances (TBARS) was completely inhibited up to 200 min in the copper-mediated system by $1 \mu\text{M}$ resveratrol, more effective than trolox or the flavonoids tested. The relevant mechanisms appeared to be a combination of copper

chelation and free radical scavenging; surprisingly, resveratrol was unable to chelate iron. In subsequent experiments it proved to be more effective than flavonoids as a chelator of copper and less effective as a free-radical scavenger (61). Resveratrol added to plasma was distributed between the lipoprotein classes according to their lipid content in the order VLDL > LDL > HDL (62). The authors' suggestion that resveratrol may be effective in a lipid as well as in an aqueous environment was supported by experiments showing that resveratrol blocked the formation of TBARS by AAPH in phospholipid liposomes. Using a different system (inhibition of cytochrome C oxidation by hydroxyl radicals generated by photolysis of H₂O₂), Turrens *et al* (41) reported that *trans*-resveratrol manifested antioxidant activity, the EC₅₀ concentration being 33 μM. These findings were extended by Fauconneau *et al* (63) who reported that *trans*-resveratrol protected rat liver microsomes against Fe²⁺-mediated lipid peroxidation and human LDL against Cu²⁺-mediated lipid peroxidation. The EC₅₀ concentrations were 3.0 and 2.6 μM, respectively: in the same range as anthocyanins but around 2-fold higher than catechins and the stilbene astringinin. The activity of resveratrol in scavenging the stable free radical DPPH (EC₅₀ 74 μM) was much higher than that of the other previously mentioned compounds.

Resveratrol appears to be a potent antioxidant in a number of other biological systems. It was more effective than vitamins C or E in preventing oxidative damage and death in a rat pheochromocytoma cell line (64). It protects the same cells against damage induced by oxidized lipoproteins (65), and also proved to be a powerful inhibitor of reactive oxygen species production in murine macrophages (Figure 3), human monocytes and human

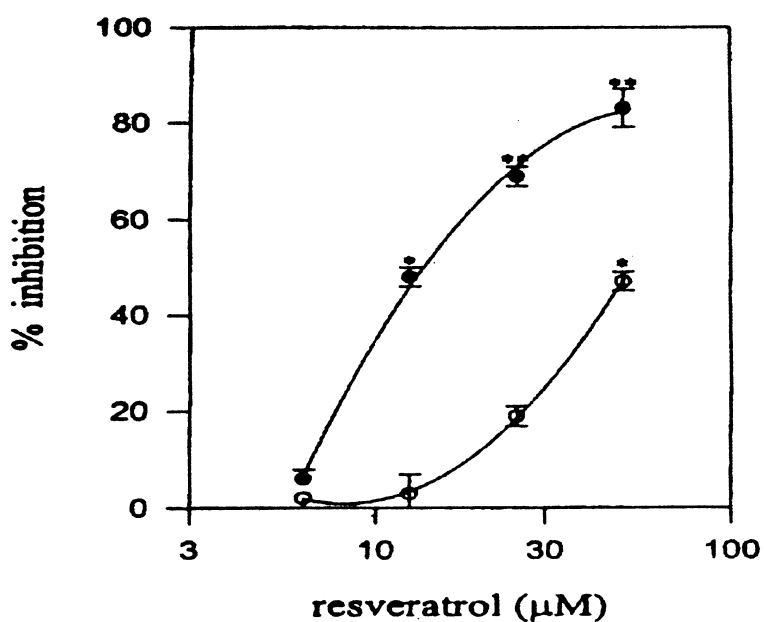


Figure 3. Inhibition by resveratrol of O₂ consumption (Solid Circles) and ROS production (Open Circles) in stimulated murine macrophages (From Ref. 66, with permission).

neutrophils, although the IC_{50} values for these effects ranged from 17 to 23 μM (66). Jang and Pezzuto (9,10) have attributed its anticancer activity in the mouse skin carcinogenesis model, at least in part, to its antioxidant properties, since phorbol ester-mediated increases in myeloperoxidase, superoxide dismutase and H_2O_2 production were restored to control levels by treatment with resveratrol. Related stilbenes also have antioxidant properties. *Cis*-resveratrol and resveratrol glucosides demonstrated protection against lipid peroxidation in mouse liver microsomes and human LDL, although the IC_{50} values were an order of magnitude greater than that of *trans*-resveratrol (63,67). Oxyresveratrol is a potent inhibitor of dopa oxidase activity (68).

VASCULAR RELAXATION AND NITRIC OXIDE PRODUCTION

Nitric oxide (NO) is produced in a wide range of cells. At least two different enzymes, nitric oxide synthase (NOS), are involved in its synthesis. One, iNOS, is inducible in response to inflammatory stimulants such as LPS in macrophages and other cells involved in inflammatory reactions. In this scenario, NO is pro-oxidant and potentially noxious. Its production by iNOS is inhibited by resveratrol in rat hepatic stellate cells (15), and macrophages (10,33,34).

The second, cNOS, is a constitutive enzyme in vascular endothelial cells. NO produced in this location prevents adherence of platelets to the endothelial surface and diffuses distally to promote relaxation of the smooth muscle layer, in part attributable to its antagonism of the vasoconstricting agent endothelin. Using rat aortic rings, Fitzpatrick *et al* (69) showed that red wine extracts were able to abolish vasoconstrictive events; a number of wine constituents (including quercetin) could reproduce this phenomenon but resveratrol was inactive in this regard. Subsequently, Chen and Pace-Asciak

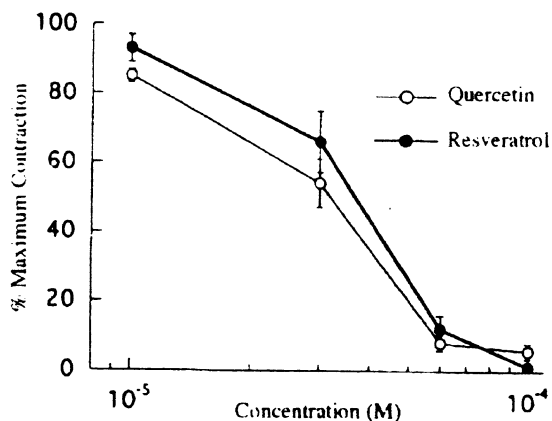


Figure 4. Relaxation of precontracted rat aortic rings by resveratrol and quercetin. (From Ref. 70, with permission).

(70) were able to demonstrate quite effective vasorelaxation by resveratrol in a similar system (Figure 4), but could not offer any explanation for the discordance between these and the previous results. A recent report (71) lends strong support to the findings of Chen and Pace-Asciak (70) by demonstrating dose-dependent inhibition by resveratrol of histamine and fluoride-induced contractions in isolated porcine coronary arteries at very low EC_{50} concentrations of <1 nmol/l.

ESTROGENIC ACTIVITY

Following the knowledge that pinosylvin, a stilbene containing one less OH group than resveratrol, is estrogenic in human breast cancer cell lines (72), Gehm et al (73) were the first to report the interaction of resveratrol with estrogen and its receptor. At 3-10 μ M concentrations, resveratrol inhibited the binding of labeled estradiol to the estrogen receptor in human breast cancer cells, and activated transcription of estrogen-responsive reporter genes transfected into these cells. This transcriptional activation was estrogen receptor-dependent, required an estrogen response element in the reporter gene, and was inhibited by specific estrogen antagonists. Depending on the cell type, it produced activation greater than, equal to or less than that of estradiol. It also increased the expression of native estrogen-regulated genes and stimulated the proliferation of an estrogen-dependent human breast carcinoma cell line.

A subsequent report described resveratrol as lacking the ability to bind to estrogen receptor in a pituitary cell line (74). Unlike other phytoestrogens tested in the same system, resveratrol did not stimulate the growth of these cells, but like them it was able to stimulate prolactin secretion by these cells in a dose and time-dependent manner. When tested in concentrations ranging from approximately 2.5-200 μ M, resveratrol inhibited the growth and proliferation of human breast cancer cell lines irrespective of their estrogen responsiveness (44). These results were extended by Lu and Serrero (75) who also grew estrogen receptor-positive breast cancer cells (MCF-7) in the presence of resveratrol. At concentrations of 1 μ M and above, the latter antagonized the growth-promoting effect of 17-beta-estradiol (1nM) upon these cells (Figure 5), as well as its stimulation of progesterone receptor gene expression. At higher concentrations (100 μ M), resveratrol blocked the expression of mRNA for TGF α and ILGF-I receptor but increased mRNA for TGF β_2 in MCF-7 cells. The increase in DNA synthesis and stimulation by resveratrol of an osteoblastic cell line was inhibited by the antiestrogen tamoxifen (11).

Although its role as a phytoestrogen has been invoked to account, at least in part, for its protection against atherosclerosis (76), recent *in vivo* experiments cast doubt about the extrapolation of these *in vitro* antiestrogenic

effects to whole animals. When given to rats by oral gavage or subcutaneous injection, resveratrol in doses ranging up to the equivalent in a 70 kg human of 2800 l of wine daily did not show any activity in an assay using uterine estrogen receptors (77). In fact, *in vitro* studies carried out by this group and reported in the same paper described the affinity of resveratrol for rat uterine estrogen receptors as 5 orders of magnitude lower than that of estradiol or diethyl-stilbestrol. Similar conclusions were reached with experiments utilizing estrogen receptor-transfected yeast cells and cos-1 cells. Finally,

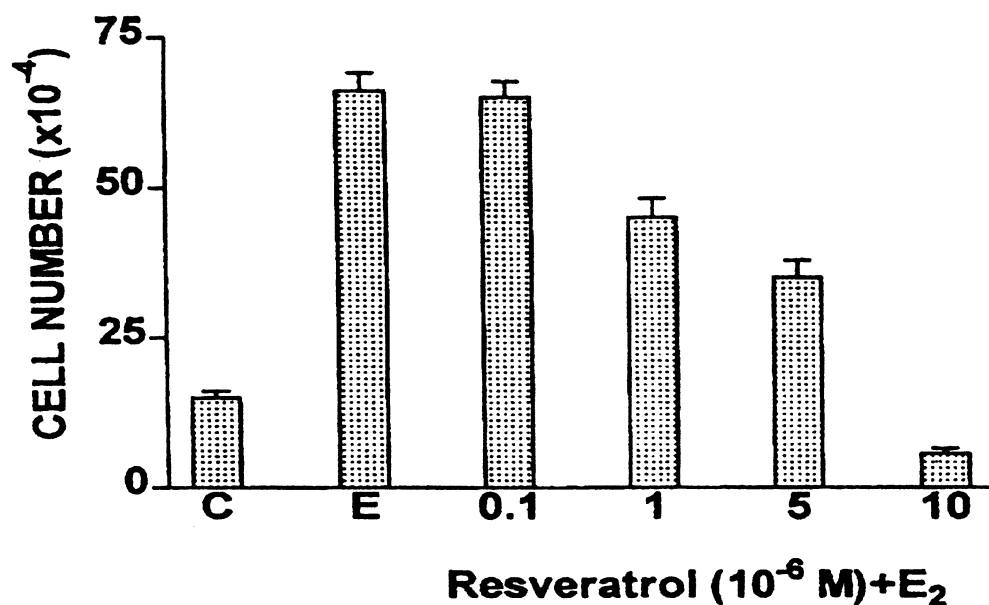


Figure 5: Antagonism by resveratrol of estrogen stimulated growth of MCF-7 human breast cells after 6 days. C, control; E, estrogen; remaining bars are various concentrations of resveratrol in presence of estrogen. (From Ref. 75, with permission).

Turner et al (78) carried out an extensive investigation on the effect of orally-administered resveratrol (graded doses equivalent to 0.5-500 ml of red wine per day for 6 days to weanling rats) upon estrogen target tissues assessed by growth rate, body weight, serum cholesterol and radial bone growth, all of which were enhanced by equivalent doses of estradiol. Resveratrol did not alter any of these parameters.

Before accepting these negative conclusions, two caveats are worthy of consideration. Firstly, it is conceivable that the affinity of resveratrol for estrogen receptors is subject to some tissue specificity, being more potent for breast than for uterus; moreover, since Ashby et al (77) had found that the affinity of resveratrol for uterine estrogen receptors was 5 orders of magnitude less than that of estradiol, it is surprising that they employed similar doses of both agonists in their whole animal experiments. Secondly, the parameters selected by Turner et al (78) may have been appropriate for estradiol, but conceivably not for resveratrol, whose widespread biological actions,

including those on cell growth and DNA synthesis, may have masked its possible role as a phytoestrogen, as judged by these criteria.

ABSORPTION AND BIOAVAILABILITY

The disappointing results of human and whole animal experiments designed to reproduce the *in vitro* actions of resveratrol give urgency to the question whether it is efficiently absorbed and if so, whether its metabolic and excretory patterns are consistent with tissue concentrations adequate to achieve desirable effects. The prerequisite to such investigations was the development of assays sensitive enough to allow the measurement of resveratrol in blood, as well as its distribution between plasma or serum and formed elements of the blood. Our group was the first to describe investigations relevant to these issues (79,80). Using a HPLC method, we found that *trans*-resveratrol added to whole human blood was >90% recoverable and partitioned as follows: serum 54.8%; erythrocytes 36.0%; leukocytes and platelets 6.1%. However, taking protein content into account, the latter fraction was the most highly enriched in resveratrol.

A subsequent report, incorrectly claiming to be the first method developed to measure resveratrol in animal and human samples, presented results that were not quite consistent with these findings (81). Washed human and rat erythrocytes, rat platelets and human LDL incorporated approximately 50%, 10% and 17.5% respectively of *trans*-resveratrol when incubated at room temperature for 15-30 min. Two HPLC methods for the measurement of plasma resveratrol have been described with detection limits of 20 µg/l (82) and 5 µg/l (83), respectively. Only the former has actually been applied in whole animals; 15 min after unspecified administration of 2 mg/kg to rats, the plasma resveratrol concentration was stated to be 175 µg/l, almost 10% of the dose given, a result completely at variance with all other literature reports on this topic (82).

Bertelli and colleagues developed a HPLC method to determine resveratrol concentrations in rat serum, and claimed a detection limit of 1ng/ml. They found that after 4 ml of red wine containing 26 µg of resveratrol given to the animals by gavage, the blood resveratrol concentration peaked around 60 min at about 15 ng/ml, or 5.8×10^{-2} percent of the dose administered, and returned to baseline by 4 hours (84). In a further experiment, they gave 13 µg of total *trans* and *cis*-resveratrol per day to a second group of rats for 15 days, at which time the concentrations of resveratrol in plasma (7.6 ng/ml), urine (66 ng/ml), heart (3 ng/ml), liver (54 ng/ml) and kidneys (44 ng/ml) were well below those required for pharmacologic activity based upon *in vitro* studies (85). A kinetic analysis revealed that relative tissue bioavailability, calculated as *Area Under the Curve* (AUC) for tissues expressed as a percentage of AUC for plasma,

accorded with the following ratios: heart, 24; liver, 218; kidneys, 295. However, they subsequently claimed that by the administration of red wine, they were able to achieve resveratrol concentrations compatible with the inhibition of platelet aggregation (86).

Table 2. Recovery of Labelled Resveratrol 24 Hours After Gastric Administration in Rats ^a

Matrix	Total (%)	Stool (%)	Urine (%)
Grape Juice	61.1 ± 4.9	10.8 ± 0.9	50.3 ± 5.7
V-8 Juice	73.6 ± 3.5	14.1 ± 1.0	59.5 ± 4.5
Alcohol	62.2 ± 2.3	13.2 ± 1.4	49.0 ± 3.1

^aMean of 8 experiments ±SEM

Our group has recently completed an investigation in which we gave 298 nCi of [³H]-resveratrol added to 10% ethanol, white grape juice or vegetable homogenate (V-8) by stomach tube to male Wistar rats (avg. weight 300g), following which the animals were held in metabolic cages for collection of urine and feces independently. After 24 hours, they were sacrificed with collection of blood, various organs, and the contents of colon and bladder that were added to stool and urine, respectively. Only traces of radioactivity were detected in the blood after 24 hours, or in groups of rats sacrificed at 30 min intervals over the first 2 hours. Urine and bladder accounted for 50-60%, and stool and colon for 11-14% of the radioactivity after 24 hours (Table 2). There were no significant differences between the three beverages. Only traces of radioactivity were detected in spleen, liver, kidney, or the cellular elements of the blood. Using ethanol as the vehicle, competition experiments were performed with cold resveratrol as well as unlabelled catechin and quercetin (two flavonoid polyphenols present in red wine that share some similar structural features with resveratrol). None of these compounds altered the amount of radioactivity in stool or urine after 24 hours. We conclude, based upon urine measurements, that around 50-60% of the *trans*-resveratrol entering the rat intestine is absorbed, probably by bulk fluid transfer rather than by receptor-mediated mechanisms, and that its clearance from the blood stream is very rapid. This percentage may be closer to 90 if we assume that all of the radioactivity not recovered in the stool was actually absorbed. 25-40% of tracer could not be accounted for and may have been deposited in adipose tissue and brain in view of its lipophilic nature. Finally, the presence of alcohol does not seem to be necessary for effective absorption to take place.

MISCELLANEOUS

Three papers have appeared that testify to efforts underway for the large-scale production of resveratrol and its glucosides, presumably for pharmaceutical purposes. Orsini *et al* have reported the synthesis of a range of resveratrol derivatives, including polydatin (piceid) by means of Wittig reactions followed by glucosylation under phase-transfer catalysis (87). Polydatin has also been generated on a preparative scale through the microbial transformation of resveratrol by a strain of *Bacillus cereus* (88). Resveratrol is much more stable than other polyphenols in grape skins and pomace after fermentation when stored at room temperature for relatively lengthy periods (89), raising the expectation that these materials will be excellent sources for its extraction and purification on a commercial scale.

An interesting set of observations was made by Busam *et al* (90) on the phenomenon of systemic acquired resistance in grapes. Treatment of cell-suspension cultures of *Vitis vinifera* with fungi or a number of chemicals caused the accumulation of resveratrol. Simultaneously, the expression of S-adenosyl-L-methionine: trans-caffeoyl-coenzyme A 3-O-methyl transferase, as well as of stilbene synthase, was increased. Phenolic esterification, in addition to the synthesis of stilbenes, may play an important role in disease resistance of grapevines.

Given the interest in the possibility that antioxidant flavonoids may prevent dementia and improve cerebral function, it is worth drawing attention to a recent paper describing an induction by resveratrol of phosphorylation of several protein kinases (mitogen-activated and extracellular signal-regulated) in differentiated and undifferentiated human neuroblastoma cells (91).

CONCLUSION

Since our first review on resveratrol (2), many more biological effects of the compound have been demonstrated *in vitro*. Further, the molecular and biochemical basis for several of these effects has been elucidated. One of the paradoxes, as true to-day as it was several years ago, is the difficulty of reproducing these biological activities in whole animals or humans. A concern at that time was the issue of whether resveratrol can actually undergo intestinal absorption. This now seems to have been affirmatively resolved. However, its excretion appears to be fairly rapid; even when quite large amounts are given, its concentrations in blood and tissues fall well below the levels required for most biological activities, and suggestions that long term administration may lead to cumulatively higher concentrations are not convincing. Given the levels in naturally-produced red wines, it seems unlikely that biologically useful concentrations will be achieved from this

source alone, although one report does provide evidence that resveratrol may be converted *in vivo* to metabolites with much greater activity (36).

Our finding that resveratrol appears to be absorbed as effectively in matrices that do not contain alcohol as in those that do lends credibility to the notion that it could feasibly be provided as a capsule or elixir, joining the rank of natural health preparations that are increasingly invading our retail outlets. Alternatively, wine makers may wake up to the realization that enriching their products with resveratrol may give them a market advantage, provided that there are no adverse organoleptic characteristics as a result. The future of resveratrol as a non-patentable natural product will depend upon the delicate interplay of scientific and commercial forces. At any time in the future the balance could change dramatically if synthetic analogues with greater potency are developed.

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