# Resveratrol Is a Potent Inhibitor of the Dioxygenase Activity of Lipoxygenase

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Resveratrol is a naturally occurring phytoalexin, present in grapes and other food products, with important antioxidant properties. Although still under debate, it is generally assumed that resveratrol has protective effects against heart diseases and probably tumor development. Lipoxygenase is a dioxygenase with peroxidase activity involved in the synthesis of mediators in inflammatory, atherosclerotic, and carcinogenic processes. Lipoxygenase activity is also involved in the generation of flavors and aromas in foods from animal or vegetal sources. The results presented here show that resveratrol was a potent inhibitor of the dioxygenase activity of lipoxygenase, with an $IC_{50} = 13\ \mu M$. Simultaneously, resveratrol was oxidized by the peroxidase activity of lipoxygenase with a $V_{max} = 0.28\ \mu M\ min^{-1}$ and a $k_M = 16.6\ \mu M$. Furthermore, oxidized resveratrol was as efficient a lipoxygenase inhibitor as in its reduced form. From the data obtained it can be concluded that both resveratrol and its oxidized form can act as inhibitors of the dioxygenase activity of lipoxygenase. In contrast, the hydroperoxidase activity of lipoxygenase was not inhibited by resveratrol. These results suggest that resveratrol may be used as an antioxidant food additive and as a pharmacological agent to prevent the generation of eicosanoids involved in pathological processes.

**Keywords:** *Resveratrol; lipoxygenase; antioxidants; co-oxidation*

## INTRODUCTION

Resveratrol (3,5,4′-trihydroxystilbene) is a phytoalexin found in grapes and other foods. This compound is produced in response to stress or pathogenic attack and has been found in a number of plant species, some of which are components of the human diet. Grapes are the fruit in which a higher content has been found, probably due to the response of *Vitis vinifera* to fungal infection. As a consequence, appreciable amounts of resveratrol can be found in wine, which has been suggested to play a role in the prevention of heart disease associated with a moderate consumption of red wine. In addition, resveratrol inhibits platelet aggregation and coagulation, alters eicosanoid synthesis, and modulates lipoprotein metabolism (Jang et al., 1997). Furthermore, because resveratrol has very weak toxicity and, at least in vitro, inhibits processes associated with cell proliferation in tumor cell lines, it was suggested that it could act as a cancer chemopreventive agent in humans (Jang et al., 1997).

Although the molecular basis for the biological activity of resveratrol is not well understood, it is known that this molecule acts as a radical scavenger, being a remarkable inhibitor of ribonucleotide reductase and DNA synthesis in mammalian cells (Fontecave et al., 1998). Likewise, it has been reported that resveratrol inhibits transcription and the activity of cyclooxygenase 2, an enzyme that catalyzes the formation of prostaglandins from arachidonic acid and plays an important role in tumorigenesis and inflammation (Subbaramaiah et al., 1998). The other enzyme involved in the oxidative metabolism of arachidonic acid is lipoxygenase. This enzyme is a stereospecific dioxygenase with hydroperoxidase activity (Kulkarni et al., 1990), which leads to the synthesis of leukotrienes and lipoxins, both compounds that mediate inflammatory responses in allergy, asthma, and arthritis. Lipoxygenase was also implicated in the formation of atherosclerotic lesions (Prigge et al., 1997) and carcinogenic processes (Furstenberger et al., 1991; Kamitani et al., 1998). Lipoxygenase activity has been involved in the biogenesis of flavor and aroma compounds in foods from animals or vegetal sources. These products could be desirable in many foods but could also give rise to off-flavors (O'Connor and O'Brien, 1991; Gata et al., 1996). As a consequence of the inhibition of lipoxygenase by naturally occurring antioxidants, the study of this process has gained research interest in the food industry and in preventive medicine fields. The results presented here indicate that resveratrol is a potent inhibitor of the dioxygenase activity of lipoxygenase, being catalytically oxidized in the presence of hydroperoxy derivatives of polyunsaturated fatty acids or hydrogen peroxide by the hydroperoxidase activity of the enzyme.

## MATERIALS AND METHODS

Resveratrol, linoleic acid, soybean lipoxygenase (type V), phenidone, and nordihydroguaiaretic acid (NDGA) were purchased from Sigma Chemical Co. All reagents used were of analytical grade.

Dioxygenase activity of lipoxygenase was determined using linoleic acid as substrate. Linoleic acid solution was prepared as in Gata et al. (1996). Lipoxygenase activity was assayed spectrophotometrically at 20 °C by following the increase in absorbance at 234 nm produced by the transformation of the *cis*,*cis*-1,4-pentadiene system of linoleic acid into the conjugated *cis*,*trans*-hydroperoxydiene derivative. Unless otherwise stated, the reaction mixture for measurement of dioxygenase activity typically contained $7.5 \times 10^{-4}$ M linoleic acid, $1 \times 10^{-9}$

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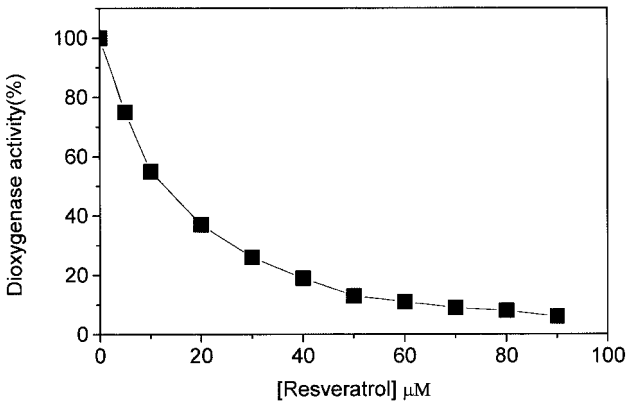


**Figure 1.** Inhibition of dioxygenase activity of soybean lipoxygenase by resveratrol. One hundred percent activity corresponds to the formation of $1.4 \times 10^{-5}$ M $min^{-1}$ hydroperoxylinoleic acid.

M soybean lipoxygenase, and 100 mM borate buffer, pH 9.0, in a final volume of 1 mL. Resveratrol oxidation by hydroperoxidase activity of lipoxygenase was determined following the decrease in absorbance at 312 nm. Resveratrol concentration was determined using an extinction coefficient of 33400 $M^{-1}$ $cm^{-1}$. This coefficient was established by measuring the absorbance at 312 nm of resveratrol solutions at known concentration, within a range of 0−100 $\mu$M prepared in 100 mM borate buffer, pH 9.0.

Hydrogen peroxide and lipoxygenase concentrations were calculated using extinction coefficients of 39.4 $M^{-1}$ $cm^{-1}$ at 240 nm and 160000 $M^{-1}$ $cm^{-1}$ at 280 nm, respectively (Cucurou et al., 1991).

RP-HPLC analyses of the resveratrol oxidation by lipoxygenase were performed using an LDC Analytical apparatus equipped with a 12.5 cm × 4 mm Lichrospher Si-60 column (5-$\mu$m particle size) (Merck, Darmstadt, Germany). Samples were eluted isocratically at 1 mL $min^{-1}$ with a solvent system of methanol/$H_2O$ (50:50). The effluent was monitored at 312 or 381 nm with an LDC Analytical 3200 Spectromonitor, and the chromatograms were registered and integrated in a Milton Roy CI 4100 computing integrator.

Oxidized resveratrol was obtained by incubating 100 $\mu$M resveratrol with lipoxygenase in the presence of 1 mM $H_2O_2$ and subsequent separation by preparative RP-HPLC. The reaction was followed spectrophotometrically at 312 nm until no change in absorbance was detected. The purity of the preparation was checked by RP-HPLC using the conditions described above.

All of the results shown in the figures and tables are the mean of at least three replicates of each experiment.

## RESULTS

As shown in Figure 1, resveratrol produced a marked concentration-dependent inhibition of lipoxygenase activity. The $IC_{50}$ obtained (13 $\mu$M) is near the $IC_{50}$ values for compounds that have been considered good lipoxygenase inhibitors such as phenidone ($IC_{50}$ = 5 $\mu$M), BW755c ($IC_{50}$ = 50 $\mu$M), or NDGA ($IC_{50}$ = 10 $\mu$M) (Cucurou et al., 1991; Papatheofanis and Lands, 1985). A Lineweaver−Burk plot (Figure 2) shows that resveratrol acts like a competitive inhibitor of the dioxygenase activity of lipoxygenase, suggesting that resveratrol displaces linoleic acid from the enzymatic site of oxidation.

When lipoxygenase was incubated at pH 9.0 with linoleic acid and a concentration of resveratrol (10 $\mu$M) that produces a 50% inhibition, a modification of the spectroscopic properties of resveratrol did occur. Repetitive scans showed an increase in absorbance (as shown in Figure 3) at 234 nm produced by the generation of

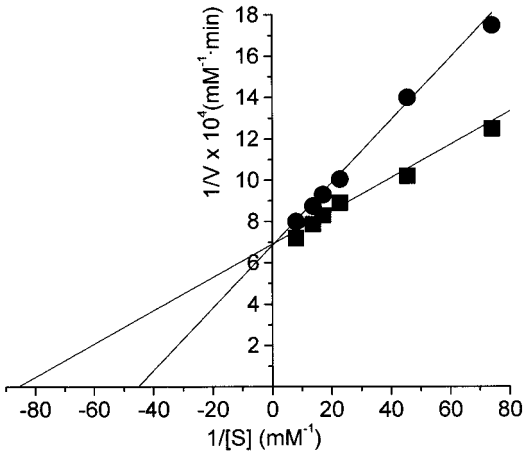


**Figure 2.** Lineweaver−Burk plot for the inhibition of soybean lipoxygenase by resveratrol. The reaction was initiated by adding $1 \times 10^{-9}$ M enzyme to the reaction mixture in the absence (squares) or presence (circles) of $1 \times 10^{-5}$ M resveratrol.

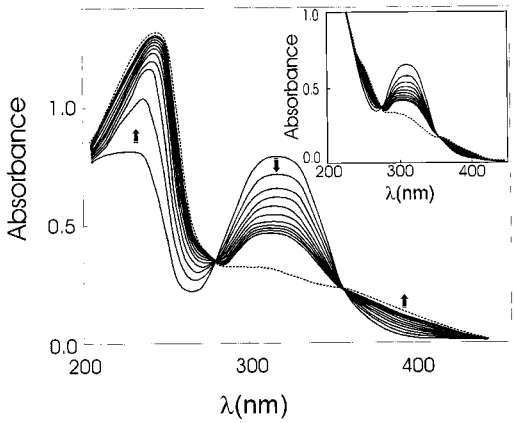


**Figure 3.** Oxidation of resveratrol by soybean lipoxygenase in the presence of linoleic acid. The reaction medium contained 100 mM borate buffer, pH 9.0, $7.5 \times 10^{-4}$ M linoleic acid, $1 \times 10^{-9}$ M lipoxygenasw, and $1 \times 10^{-5}$ M resveratrol. The elapsed time between scans was 30 s. The dotted line corresponds to the spectrum recorded at 10 min of incubation. (Inset) Oxidation of resveratrol by soybean lipoxygenase in the presence of $H_2O_2$.

hydroperoxy derivatives from linoleic acid and a strong decrease in the absorption band centered at 318 nm. Simultaneously, an increase in absorbance in the 390−400 nm region was found. It should be noted that the presence of two isosbestic points, at 275 and 354 nm, suggests that only two species, resveratrol and its oxidized form, are involved in the course of the reaction (Poole and Bashford, 1987). The effect of resveratrol on the hydroperoxidase activity of lipoxygenase exhibited a hyperbolic shape (Figure 4). This kinetic showed that resveratrol oxidation and dioxygenase activity of lipoxygenase were linearly correlated (inset of Figure 4), suggesting that oxidized resveratrol may be involved in the inactivation process. Resveratrol oxidation was found to be dependent on the amount of lipoxygenase used in the assay, but independent of the hydroperoxide concentration (Table 1). To confirm the implication of hydroperoxidase activity of lipoxygenase in the oxidation of resveratrol, we incubated the enzyme with resveratrol in the presence of $H_2O_2$, obtaining a spectral pattern similar to that obtained when linoleic acid was used as substrate (see inset of Figure 3). The chromatographic analyses by RP-HPLC (see Materials and

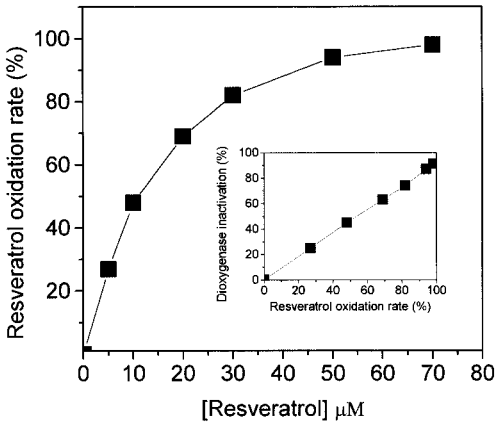


**Figure 4.** Effect of resveratrol concentration on the hydroperoxidase activity of lipoxygenase. Resveratrol was incubated in the presence of $7.5 \times 10^{-4}$ M linoleic acid and $1 \times 10^{-9}$ M lipoxygenase The oxidation rate was calculated from the decrease in absorbance at 312 nm. One hundred percent oxidation resveratrol rate corresponds to 0.28 $\mu$M min$^{-1}$. (Inset) Correlation between dioxygenase inactivation and resveratrol oxidation rate. Dioxygenase activity was determined from the increase in absorbance at 234 nm.

**Table 1. Dependence of Rate of Oxidation of Resveratrol on Concentrations of Lipoxygenase and LOOH**

| [E] ($\mu$g/mL) | [LOOH][a] (M) | resveratrol oxidation[b] (M min$^{-1}$) |
|---|---|---|
| 0.4 | $1.7 \times 10^{-6}$ | $5.5 \times 10^{-7}$ |
| 0.2 | $1.7 \times 10^{-6}$ | $2.6 \times 10^{-7}$ |
| 0.4 | $3.4 \times 10^{-6}$ | $5.4 \times 10^{-7}$ |

[a] Hydroperoxide from linoleic acid was prepared by incubation of soybean lipoxygenase with linoleic acid. The products of the reaction were purified by RP-HPLC using methanol/water (75:25) as solvent system. The concentration of LOOH was calculated from $A_{234}$ by using an extinction coefficient of 25000 M$^{-1}$ cm$^{-1}$ (Kemal et al., 1987). [b] Resveratrol oxidation was calculated from the decrease in absorbance at 312 nm by using an extinction coefficient of 33400 M$^{-1}$ cm$^{-1}$ (see Materials and Methods).

Methods) of resveratrol oxidation products using linoleic acid or $H_2O_2$ as substrate were coincident. Figure 5 shows the RP-HPLC elution profile for resveratrol at 0 and 10 min of incubation in the presence of lipoxygenase and hydrogen peroxide. It can be seen that the peak with a retention time of 5 min, detected at 312 nm, disappeared at the end of the incubation period. Simultaneously, a peak centered at 1.5 min, corresponding to the oxidized molecule of resveratrol, was detected at 390 nm at the end of the incubation period. Likewise, the UV−vis spectrum of the product obtained by lipoxygenase-catalyzed oxidation of resveratrol in the presence of $H_2O_2$ (see inset of Figure 3) is coincident with that obtained for the same reaction but in the presence of linoleic acid.

Resveratrol oxidation was abolished when NDGA, one of the most efficient lipoxygenase inhibitors known (Kemal et al., 1987), or dithizone, a potent lipoxygenase inhibitor that forms strong complexes with $Fe^{3+}$ (Pistorius and Axelrod, 1974), was used. When lipoxygenase was incubated in the presence of linoleic acid, 10 $\mu$M resveratrol, and 0.11 mM NDGA, inhibition of both dioxygenase and hydroperoxidase activities was produced and, as a consequence, resveratrol was not oxidized. The same effect was observed in the presence of 4 $\mu$M dithizone. In addition, no oxidation of resveratrol was observed when boiled enzyme (5 min at 100 °C) was used in the assay of activity. Taken together, these results strongly suggest the enzymatic nature of resveratrol oxidation in our experimental conditions.

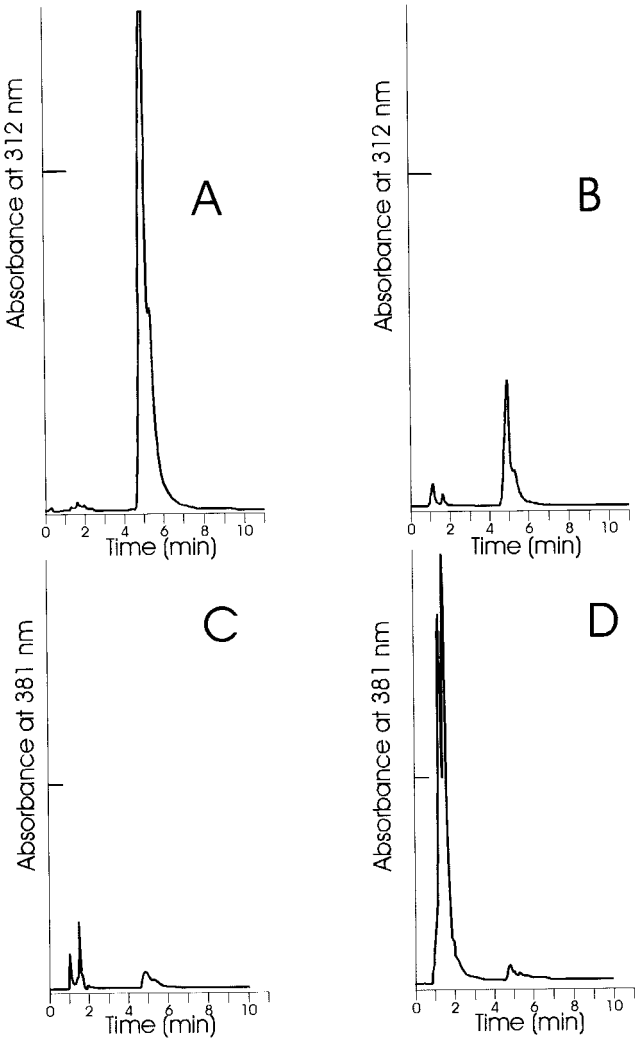


**Figure 5.** RP-HPLC analysis of the oxidation of resveratrol by lipoxygenase: $4 \times 10^{-5}$ M resveratrol was incubated in the presence of $1 \times 10^{-3}$ M hydrogen peroxide and $1 \times 10^{-9}$ M lipoxygenase in 100 mM borate buffer, pH 9.0. The reaction was started by the addition of enzyme and stopped at $t = 0$ min (A and C) or at $t = 10$ min (B and D) by addition of ethanol. Sample was loaded in the RP-HPLC column by direct injection, using a solvent system of methanol/$H_2O$ (50:50).

To check if the product of the enzymatic oxidation of resveratrol caused the inhibition of the dioxygenase activity of lipoxygenase, incubations of the enzyme in the presence of linoleic acid were performed in the presence of several concentrations of oxidized resveratrol (see Materials and Methods). The results obtained (Figure 6) clearly indicated a strong inhibition of the dioxygenase activity by this compound, with an $IC_{50}$ that was coincident with the value obtained by incubation in the presence of reduced resveratrol (Figure 1).

## DISCUSSION

There is considerable interest in the development of inhibitors of lipoxygenases. In mammals, hydroperoxides play an important role in immune response and in the inflammatory process, and they have been recently implicated in the formation of atherosclerotic lesions. As a consequence, there is considerable pharmacological interest in the study of compounds that inhibit lipoxygenase activity (Prigge et al., 1997). On the other hand, lipoxygenase plays an important role in the mechanism

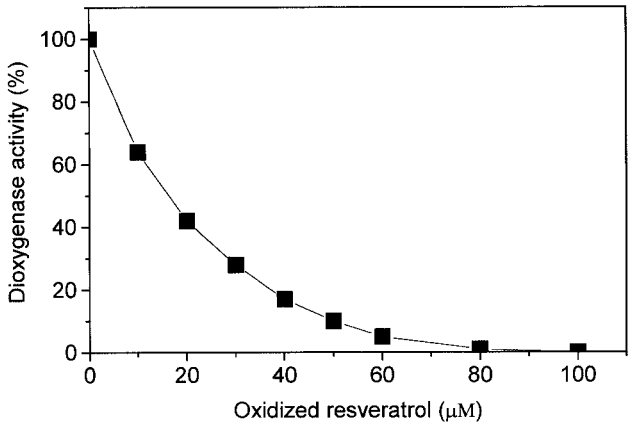


**Figure 6.** Effect of oxidized resveratrol on dioxygenase activity of lipoxygenase. Oxidized resveratrol was obtained by incubating resveratrol in the presence of lipoxygenase and $H_2O_2$. One hundred percent dioxygenase activity corresponds to $1.4 \times 10^{-5}$ M $min^{-1}$ of hydroperoxylinoleic acid formation.

of lipid oxidation in food, being responsible for generating off-flavors in certain food system. For these reasons the use of natural antioxidants in the food industry has shown a rapid increase in the past few years (Ramarathnam et al., 1995).

Recently, it has been reported that resveratrol, a usual constituent of the human diet, inhibits cyclooxygenase activity and possesses anti-inflammatory effects (Subbaramaiah et al., 1998). Although aspirin and other nonsteroidal anti-inflammatory compounds are potent inhibitors of cyclooxygenase, no effective pharmacological inhibitor of lipoxygenase is presently available. In this work we show, for the first time, that resveratrol is a remarkable lipoxygenase inhibitor with a potential use as a pharmacological and food additive agent.

The data presented here reveal an inhibitory potency of resveratrol on lipoxygenase activity similar to that found for more potent redox inhibitors of lipoxygenase such as phenidone, BW755c, and NDGA. It is important to note that these compounds do not have clinical or food additive uses due to their toxicity to humans (McMillan and Walker, 1992). In contrast, no signs of toxicity have been detected for resveratrol (Jang et al., 1997).

Our results suggest that resveratrol and its oxidized form, obtained by the hydroperoxidase activity of lipoxygenase, are efficient inhibitors of lipoxygenase activity. It is well-known that lipoxygenase inactivation by catecholic or pirazoline derivatives occurs only after oxidation of these compounds by the hydroperoxidase activity of lipoxygenase (Cucurou et al., 1991; Kemal et al., 1987). The fact that the maximum rate of resveratrol oxidation was obtained at a concentration of inhibitor at which dioxygenase activity was abolished suggests that the dioxygenase and hydroperoxidase activities of lipoxygenase are independent. Our results revealed that resveratrol is not an irreversible redox inhibitor of lipoxygenase. NDGA, a well-known lipoxygenase inhibitor, produces the reduction of the enzyme−$Fe^{3+}$ complex (active) to the enzyme−$Fe^{2+}$ form (inactive), concomitant with the inhibition of both dioxygenase and hydroperoxidase activities (Kemal et al., 1987). Resveratrol, in contrast, acted like a reducing agent, converting lipoxygenase to its ferrous form. This reduced form is then turned back to its active form by fatty acid hydroperoxides or by $H_2O_2$. The fact that oxidized and reduced resveratrol exhibited close $IC_{50}$ values strongly suggests that oxidized resveratrol can interact with the active site of the enzyme with the same efficiency as the reduced molecule, resulting in competitive inhibition of the dioxygenase activity. This inhibition did not block the redox cycle and, as a consequence, hydroperoxidase activity was maintained. In addition, the relatively low $V_{max}$ value for resveratrol oxidation (0.28 $\mu$M $min^{-1}$) eliminates the possibility that the inhibitory effect on dioxygenase activity by resveratrol, as shown in Figure 1, could be due to accumulation of the oxidized molecule in the reaction medium.

The mechanism proposed, in which resveratrol inhibits the dioxygenase but not the hydroperoxidase activity of lipoxygenase, suggests that resveratrol could be of great relevance in mammals, in particular considering that the dioxygenase activity of lipoxygenase is involved in numerous pathological processes and that hydroperoxidase activity plays an important role in xenobiotic detoxification (Kulkarni et al., 1988). In addition, the use of resveratrol as a food additive may be considered to prevent undesired off-flavors derived from the enzymatic activity of lipoxygenase in food products.

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## LITERATURE CITED

Cucurou, C.; Battioni, J. P.; Thang, D. C.; Nam, N. H.; Mansuy, D. Mechanisms of inactivation of lipoxygenases by phenidone and BW755c. *Biochemistry* **1991**, *30*, 8964−8970.

Fontecave, M.; Lepoivre, M.; Elleingand, E.; Gerez, C.; Guittet, O. Resveratrol, a remarkable inhibitor of ribonucleotide reductase. *FEBS Lett* **1998**, *421*, 277−279.

Furstenberger, G.; Hagedorn, H.; Jacobi, T.; Besemfelder, E.; Lehman, W. D.; Marks, F. Characterization of an 8-lipoxygenase activity induced by the phorbol ester tumor promoter 12-O-tetradecanoylphorbol-13-acetate in mouse skin in vivo. *J. Biol. Chem.* **1991**, *266*, 15738−15745.

Gata, J. L.; Pinto, M. C.; Macías, P. Lipoxygenase activity in pig muscle: purification and partial characterization. *J. Agric. Food Chem.* **1996**, *44*, 2573−2577.

Jang, M.; Cai, L.; Udeani, G. O.; Slowing, K. V.; Thomas, C. F.; Beecher, C. W. W.; Fong, H. H. S.; Farnsworth, N. R.; Kinghorn, A. D.; Mehta, R. G.; Moon, R. C.; Pezzuto, J. M. Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. *Science* **1997**, *275*, 218−220.

Kamitani, H.; Geller, M.; Thomas, E. Expresion of 15-lipoxygenase by human colorectal carcinoma Caco-2 cells during apoptosis and cell differentiation. *J. Biol. Chem.* **1998**, *273*, 21569−21577.

Kemal, C.; Flamberg, P. L.; Krupinski-Olsen, R.; Shorter, A. Reductive inactivation of soybean lipoxygenase 1 by catechols: A possible mechanism for regulation of lipoxygenase activity. *Biochemistry* **1987**, *26*, 7064−7072.

Kulkarni, A. P.; Mitra, A.; Chaudhuri, J.; Byczkowski, J.; Richards, I. Hydrogen peroxide: A potent activator of dioxygenase activity of soybean lipoxygenase. *Biochem. Biophys. Res. Commun.* **1990**, *166*, 417−423.

McMillan, R. M.; Walker, E. R. Designing therapeutically effective 5-lipoxygenase inhibitors. *Trends Pharmacol. Sci.* **1992**, *13*, 323−330.

O'Connor, T. P.; O'Brien, N. M. Significance of lipoxygenase in fruits and vegetables. In *Food Enzymology*; Fox, P. F., Ed.; Elsevier: Amsterdam, The Netherlands, 1991; Chapter 8.

Papatheofanis, F. J.; Land, W. E. M. Lipoxygenase mechanism. In *Biochemistry of Arachidonic Acid Metabolism*; Lands, W. E. M., Ed.; Martinus Nijhoff Publishing: Boston, MA, 1985; pp 9−39.

Pistorius, E. K.; Axelrod, B. Iron, an essential component of lipoxygenase. *J. Biol. Chem*. **1974**, *249*, 3183−3186.

Poole, R. K.; Bashford, C. L. Spectra. In *Spectrophotometry & Spectrofluorimetry*; Harris, D. A., Bashford, C. L., Eds.; IRL Press: Oxford, U.K., 1987; pp 23−48.

Prigge, S. T.; Boyington, J. C.; Faig, M.; Doctor, K. S.; Gaffney, B. J.; Amzel, L. M. Structure and mechanism of lipoxygenases. *Biochimie* **1997**, *79*, 629−636.

Ramarathnam, N.; Osawa, T.; Ochi, H.; Kawakishi, S. The contribution of plant food antioxidants to human health. *Trends Food Sci. Technol.* **1995**, *6*, 75−82.

Subbaramaiah, K.; Chung, W. J.; Michaluart, P.; Telang, N.; Tanabe, T.; Inoue, H.; Jang, M.; Pezzuto, J. M.; Dannenberg, A. J. Resveratrol inhibits cyclooxygenase-2 transcription and activity in phorbol ester-treated human mammary epithelial cells *J. Biol. Chem*. **1998**, *273*, 21875−21882.

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