Potent Antioxidative Activity of Vineatrol®30 Grapevine-shoot Extract

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The health promoting effects of a grapevine-shoot extract named Vineatrol®30, which contains resveratrol (Resv) as well as considerable amounts of Resv oligomers, have recently been investigated. In the present study, we analyzed the free radical scavenging capacity, the ability to inhibit lipid peroxidation, and the capacity to enhance the human glutathione peroxidase 1 (GPx) and the human superoxide dismutase 1 (SOD) gene promoter activities of Vineatrol®30. Vineatrol®30 was able to scavenge the 2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonic acid radical cation and led to concentration-dependent inhibition of lipid peroxidation, Vineatrol®30 not being superior to Resv alone in both cases. Vineatrol®30 also enhanced the gene promoter activities of human GPx and SOD expressed in V79 cells, whereas this effect could not be demonstrated for Resv. In summary, the results presented in this study show that the Vineatrol®30 grapevine-shoot extract is a free radical scavenger and potent antioxidant at non-cytotoxic concentrations.

Key words: antioxidative activity; glutathione peroxidase; resveratrol; resveratrol oligomer; superoxide dismutase

In recent years, the possible benefits of consuming low to moderate amounts of alcoholic beverages, particularly red wine, for the prevention of coronary heart disease (CHD) has received increasing attention. The term “French paradox” is used to point out that people living in certain parts of France, where red wine is traditionally consumed during meals, show a low mortality risk due to CHD, despite having a similar lifestyle to people living in USA and many other developed countries which are known to have a comparative high risk of suffering from CHD. trans-3,5,4′-Trihydroxystilbene (resveratrol, Resv) is thought to be one of the main red wine components being responsible for a number of CHD protective effects, including the reduction of myocardial damage during ischemia-reperfusion, modulation of vascular cell functions, inhibition of low-density lipoprotein oxidation and suppression of platelet aggregation.1

Many of the cardioprotective effects of Resv are due to its antioxidative activity, which has been extensively documented in the past.2–5 In the meantime, it is known that so-called Resv oligomers are also present in wine and display similar or even higher antioxidative activity than Resv itself. In this context, it has been shown that e-viniferin is able to scavenge superoxide ions6 and to inhibit lipid peroxidation in rat liver microsomes.7 Furthermore, piceatannol protects against hydrogen peroxide-induced DNA damage in leukaemia cell lines8 and reduces the level of chemically induced reactive oxygen and nitrogen species in PC-12 cells.9

In the last few years, the health-promoting effects of a grapevine-shoot extract named Vineatrol®30, which contains Resv as well as a considerable amount of resveratrol oligomers, has been investigated. On the one hand, it has been shown that Vineatrol®30 exerts antiproliferative and pro-apoptotic effects when added to a chronic B lymphocytic leukaemia cell culture and inhibits the proliferation of human colon carcinoma cells by inhibiting cell cycle progression.11 On the other hand, it has been demonstrated that Vineatrol®30 protects against kainic acid-induced seizures in rats, this effect being accompanied by a reduced level of malondialdehyde and reduced expression of heat shock protein 72.12

The individual polyphenols present in a complex polyphenol mixture such as Vineatrol®30 may act synergistically and therefore elicit a stronger health-promoting effect than Resv alone. This has been demonstrated when comparing the antiproliferative effects of Vineatrol®30 and Resv in a chronic B lymphocytic leukaemia cell culture.10 In the present study, we tested whether this would also be the case when comparing the antioxidative activity of

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Abbreviations: ABTS, 2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonic acid; CHD, coronary heart disease; GPx, glutathione peroxidase 1; Querc, quercetin; Resv, resveratrol; SOD, superoxide dismutase 1; TEAC, Trolox equivalent antioxidant concentration; Trol, Trolox
Vineatrol® 30 and Resv. The biological factors analyzed were the free radical scavenging capacity, the ability to inhibit lipid peroxidation, and the capacity to enhance the gene promoter activities of human glutathione peroxidase (GPX) and human superoxide dismutase (SOD).

Materials and Methods

**Chemicals.** Trolax (Trol), queretin (Querc), Resv and thiorbarbituric acid were purchased from Sigma-Aldrich (Steinheim, Germany), 2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) from Roche (Mannheim, Germany), and the Alamar Blue™ stock solution from Morphosys ASD (Düsseldorf, Germany). The Vineatrol® 30 grapevine-shoot extract was kindly provided by Breko (Bremen, Germany) and Actichem (Montauban, France) who develop and produce this extract in cooperation. Vineatrol® 30 contained 15.2% Resv, 13.2% e-viniferin, 4.4% ampeolpin A, 2.8% hopcaepalin, 2.1% iso-trans-viniferin, 1.9% vitisin A, 1.9% vitisin B, 1.8% piceatannol and 1.6% miyabenol C. Stock solutions of Trol, Querc, Resv and Vineatrol® 30 were prepared in dimethyl sulfoxide.

**ABTS assay.** The free radical scavenging activity of Trol, Resv and Vineatrol® 30 was measured by a modified version of the ABTS radical cation decolorization assay originally described by Re et al.†13 The stable green ABTS radical cation was prepared one day before performing the assay by reacting 7 mM ABTS with 2.45 mM potassium phosphate buffer pH 7.4. The absorbance at 734 nm was then determined. Trol, a vitamin E derivative, was used in this assay as a reference compound. The absorbance of the ABTS radical cation was determined. Trol, a vitamin E derivative, was used in this assay as a reference compound. The absorbance of the ABTS radical cation was determined. Trol, a vitamin E derivative, was used in this assay as a reference compound.

**Cell culture.** The Chinese hamster fibroblast cell line, V79, was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). Cells were grown in Dulbecco’s modified Eagle’s medium (Biochrom, Berlin, Germany) supplemented with 10% foetal calf serum, 100 𝉮/ml of streptomycin and 100 𝉮/ml of penicillin (Biochrom) at 37 °C under a humidified 5% CO₂ atmosphere.

**Cytotoxicity assay.** Five thousand V79 cells in 200 𝜇l of the cell culture medium were seeded in each well of a 96-well plate and cultured at 37 °C for 24 h. The cell culture medium was then replaced by a fresh medium with an increasing amount of the compound/extract, and the cells were incubated for another 24 h at 37 °C. Each concentration tested was performed in quadruplicate. The cell culture medium was then removed, the cells rinsed once with warm PBS, and 200 𝜇l of the cell culture medium with 5% v/v Alamar Blue™ was added to each well. Blank fluorescence values were measured immediately after adding the Alamar Blue™ (excitation wavelength: 530 nm; emission wavelength: 590 nm). Following an incubation period of 3 h, a second measurement of fluorescence was performed. The blank values were subtracted from the fluorescence values measured after 3 h, and the relative cytotoxicity was calculated according to the following equation: % cytotoxicity = [(Fsolvent – Ftest compound)/Fsolvent] × 100.

**Dual-luciferase® reporter gene assay.** The plasmids used in the present study and subsequently mentioned below have been described in detail elsewhere.†13 Eight thousand V79 cells in 200 𝜇l of the cell culture medium were seeded in each well of a 96-well plate and cultured for 24 h. Thereafter, the cells were transfected according to Ullmann et al.††14 with two modifications. First, 0.5 𝜇g of plasmid pGL3-SOD with the human SOD1 promoter or pGL3-GPx with the GPx1 promoter and 0.05 𝜇g of plasmid pGL4-TK as an internal standard were transfected into the V79 cells by using a CellPhect transfection kit from GE-Healthcare (Munich, Germany), whereby 1.6 𝜇l of plasmid DNA (pGL3-SOD or pGL3-GPx + pGL4-TK), 1.6 𝜇l of buffer A of the above-mentioned kit and 3.2 𝜇l of buffer B of this kit were added to each well. Second, the cells were transfected for 16 h at 37 °C. The subsequent glycerol shock, treatment with the test compounds, cell lysis, measurement of the Firefly and Renilla luciferase activities in 20 𝜇l of the cell lysate and calculation of the induction of Firefly luciferase activity by the test compounds was performed as described by Ullmann et al.†5

**Statistical analysis.** The results were subjected to an analysis of variance and subsequent Dunnett multiple-comparison test with SPSS version 11 software for Windows. Differences between the single groups were tested by performing an analysis of variance and Tukey’s post hoc test.

**Results**

In order to determine the antioxidative potential of Vineatrol® 30 and to compare it with that of Resv, the ability of Resv and Vineatrol® 30 to scavenge the ABTS radical cation was determined. Trol, a vitamin E derivative, was used in this assay as a reference compound and was assayed at the same concentrations as those for Resv and Vineatrol® 30. The two compounds and the extract led to a concentration-dependent reduction in the absorbance of the ABTS radical cation (Fig. 1A), whereby the reduction was more pronounced in the case of Resv than that of Vineatrol® 30 at a concentration up to 2.5 𝜇g/ml. At higher concentrations the maximal reduction in the absorbance of the ABTS...
radical cation was achieved by all three compounds. In the second step, the Trolox equivalent antioxidative concentration (TEAC) values were calculated. As shown in Fig. 1B, Resv resulted in 3.3-fold higher antioxidative activity than Trol.

Further evidence for the antioxidative potential of a test compound is its ability to protect biological macromolecules such as lipids against oxidative stress. Therefore, the inhibition of lipid peroxidation by Resv and Vineatrol®30 was tested. As shown in Fig. 2, Resv and Vineatrol®30 led to concentration-dependent inhibition of lipid peroxidation. However, the dose-response curve for Vineatrol®30 was less steep than that for Resv; at concentrations of 1.6 and 2.3 μg/ml, the difference between the two experimental groups reached statistical significance. Moreover, Resv reached maximal lipid peroxidation inhibition at a concentration of 2.3 μg/ml, whereas Vineatrol®30 reached it at a concentration of 4.6 μg/ml.

In order to test the capacity of Resv and Vineatrol®30 to enhance the gene-promoting activities of human GPx and human SOD expressed in V79 cells, a recently established reporter gene assay was used. Querc (10 μM) was included as a positive control and, as previously shown by Ullmann et al., clearly induced both the SOD and GPx gene-promoting activities (Fig. 3). A 24-h incubation period with 2.3 μg/ml of Vineatrol®30 caused 1.45- and 1.56-fold increases of the SOD and GPx gene-promoting activities, respectively (Fig. 3), both activities in the Vineatrol®30-treated cells being (from a statistical point of view) significantly higher than those in the solvent-treated cells. Incubation of the cells with 9.1 μg/ml or 18.3 μg/ml of Vineatrol®30 did not lead to any further increase in the SOD and GPx gene-promoting activities, respectively, and the induction factors actually tending to decrease with increasing concentration of Vineatrol®30. While a concentration of 1.1 (4.8 μM) or 2.3 μg/ml (10 μM) of Resv did not result in a statistically significant increase of the SOD and GPx gene-promoting activities, 4.6 μg/ml (20 μM) of Resv actually led to a 20–25% decrease in the SOD and GPx gene-promoting activities when compared to the control cells.

One possible explanation for the lack of induction of the SOD and GPx gene-promoting activities with increasing Resv and Vineatrol®30 concentrations could be that they became cytotoxic at higher concentrations.
In order to test this hypothesis, an Alamar Blue/CytoTox-96 cytotoxicity assay was performed. As shown in Fig. 4, Resv and Vineatrol/Cytotoxicity assay was performed. As shown in Fig. 4, Resv and Vineatrol/C210 exerted a concentration-dependent cytotoxic effect on V79 cells, the effect being much more pronounced with Resv than with Vineatrol/C210. In the case of Vineatrol/C210, a concentration of 2.3 µg/ml was not cytotoxic at all, whereas concentrations of 4.6, 9.1, 13.8, 18.3 and 22.8 µg/ml led to cytotoxicity levels of 4, 7, 8, 13 and 22%, respectively (Fig. 4). Resv at concentrations of 2.3, 4.6, 9.1, 13.8, 18.3 and 22.8 µg/ml resulted in cytotoxicity levels of 6.5, 11, 22, 27, 36 and 46%, respectively (Fig. 4). The decrease in cell viability became statistically significant after incubating the cells with ≥ 18.3 µg/ml of Vineatrol/C210 and with ≥ 9.1 µg/ml of Resv.

**Discussion**

Resv is known to be a strong antioxidant and free radical scavenger,2–5) and its antioxidative activity is considered to be essential in the prevention of chemi-

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**Fig. 2.** Lipid Peroxidation Inhibition by Resv and Vineatrol/C210.
Each result is expressed as the mean ± standard deviation of lipid peroxidation inhibition (i.e., the quotient between the absorbance of the thiobarbituric acid-malondialdehyde complex formed in the presence of a test compound dissolved in DMSO and the absorbance of the complex formed in the presence of DMSO alone times 100). The individual concentrations of Resv and Vineatrol/C210 tested were 0.2, 0.7, 1.6, 2.3, 4.6, 9.1, 13.8, 18.3 and 22.8 µg/ml. The data were obtained from four independent experiments, the incubation with each test compound being performed in 4 different wells per experiment. a, Value significantly different from the corresponding solvent control value (p ≤ 0.05). b, Value for Resv significantly different from the corresponding Vineatrol/C210 value (p ≤ 0.05).

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**Fig. 3.** Induction of SOD and GPx Promoter Activities by Querc, Resv and Vineatrol/C210.
Each result is expressed as the mean ± standard deviation of the relative SOD and GPx promoter activities (i.e., the quotient between the SOD/GPx promoter activity in the presence of a test compound dissolved in DMSO and the SOD/GPx promoter activity in the presence of DMSO alone times 100). The data were obtained from three independent experiments, the incubation with 10 µM Querc, 2.3 µg/ml (10 µM) of Resv or 2.3 µg/ml of Vineatrol/C210 being performed in 4 different wells per experiment. The black dot indicates significant difference from the solvent control value (100%; p < 0.05).

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**Fig. 4.** Cytotoxic Effect of Resv and Vineatrol/C210 on V79 Cells.
Each result is expressed as the mean ± standard deviation of the decrease in cell viability (i.e., the quotient between the cytotoxicity observed in Resv- or Vineatrol/C210-treated cells and that in solvent-treated cells times 100). The data were obtained from four independent experiments, the incubation with each test compound being performed in 4 different wells per experiment. a, Value significantly different from the corresponding solvent control value (p ≤ 0.05). b, Value for Resv significantly different from the corresponding Vineatrol/C210 value (p ≤ 0.05).
cally induced cancer by inhibiting the initiation step of tumour development. In recent years, natural Resv oligomers such as $\varepsilon$-viniferin and piceatannol with even higher antioxidative activity than Resv have been identified. Vineatrol, a grapevine shoot extract including Resv, $\varepsilon$-viniferin, amelopsin A, hopeaphenol, iso-trans-$\varepsilon$-viniferin, vitisin A, vitisin B, piceatannol and miyabenol C, exhibits antiproliferative activity against human chronic B lymphocytic leukaemia, human colon carcinoma and human hepatoma cells, thereby showing a stronger effect than Resv itself. While the antiproliferative activity of Vineatrol has been documented, its antioxidative capacity had not previously been analyzed in detail. Taking into account that the extract used in the present study contained Resv and Resv oligomers, we speculated that the individual polyphenols present in Vineatrol could act synergistically and therefore elicit a stronger antioxidative effect than Resv alone.

The results obtained in the present study show that Vineatrol was able to scavenge the ABTS radical cation and to inhibit lipid peroxidation, but that it did not elicit a stronger antioxidative effect than Resv alone. Interestingly, Vineatrol also enhanced the gene-promoting activities of human GPX and human SOD expressed in V79 cells, whereas this effect could not be demonstrated with Resv alone. The fact that the GPX and SOD gene-promoting activities did not increase (in the case of Vineatrol) or even decreased (in the case of Resv) with increasing concentration of Resv and Vineatrol was most probably due, as shown in the present study, to the cytotoxicity displayed by Resv and Vineatrol at higher concentrations. In this context, it should be noted that the cytotoxic effect of Resv and Vineatrol was observed at concentrations within the range of those previously having been shown to inhibit the growth of human tumour cells held in culture.

In the case of Vineatrol an alternative explanation could be that it included certain compounds with the ability to repress the gene-promoting activities, this effect being first seen if these compounds were present at a certain (high) concentration in the wells.

This study shows that the individual polyphenols present in Vineatrol did not act synergistically with Resv. One could hypothesize that this was due to compounds having pro-oxidative activity in the extract. However, up to the present time, analytical procedures have failed to identify such compounds. Alternatively, one could argue that, under certain circumstances (e.g., at a high concentration), individual polyphenols in Vineatrol could act as pro-oxidants and lead to oxidative stress. However, no evidence for the induction of oxidative stress by Vineatrol has been presented in previous studies, and in this study, control incubations of a rat brain homogenate with Vineatrol alone did not result in the induction of lipid peroxidation (C. Müller, unpublished observations). Hence, the data obtained until now do not lend support to the hypothesis that Vineatrol by itself or any individual polyphenol in the extract possessed pro-oxidative activity and was able to induce oxidative stress.

If one takes into account that the concentration of Resv in many red wines ranges between 0.5 and 10 $\mu$g/ml and that the bioavailability of free Resv in humans is extremely low (1–2% at the most), the amount of Resv used in the different assays of the present study was within the concentration range of Resv taken up when drinking one glass of red wine (125 ml) per day. In the case of $\varepsilon$-viniferin, such a calculation cannot be made, since data on its bioavailability in humans are not available.

The effect of the natural flavonoid, Querc, on the expression of the antioxidative enzymes, GPX and SOD, has been studied in a variety of test systems in vivo and in vitro, and these studies have yielded contradictory results. Breinholt et al. have reported that gavage administration of Querc to rats for two weeks decreased the GPX activity in erythrocytes, whereas Rahraman and Inai have shown that Querc prevented the decrease of GPX and SOD activities in erythrocytes and skin of rats irradiated with ultraviolet A light. Furthermore, in the rat hepatoma cell line, 44IE, 5–100 $\mu$M Querc reduced the GPX and SOD mRNA levels. In the human hepatoma cell line, Hep2, 0.1–1 $\mu$M Querc led to an increase in GPX level and to a decrease in SOD mRNA level, while 10 $\mu$M Querc enhanced the SOD mRNA level. Since 10 $\mu$M Querc had led to enhanced GPX and SOD gene-promoting activities in V79 cells in a very recent study by Ullmann et al., it was used as a “positive” control in the present report. As expected, 10 $\mu$M Querc led to a clear increase in GPX and SOD gene-promoting activities. The magnitude of the increase in the GPX and SOD gene-promoting activities elicited by Vineatrol (about 1.5-fold) is similar to that of 10 $\mu$M Querc and is also within the range of the increases previously observed with such other natural products and extracts as ginsenoside Rb2, Mauritian endemic plant extracts and genistein.

Taken together, the results show that scavenging of the ABTS radical cation, inhibition of lipid peroxidation, and increase in GPX and SOD gene-promoting activities by Vineatrol occurred at a non-cytotoxic concentration level of the grapevine-shoot extract, this being the great advantage of Vineatrol over Resv alone. In the case of a Resv concentration not leading to cytotoxicity (i.e., $\leq$ 4.6 $\mu$g/ml), this resulted in a strong free radical scavenging and lipid peroxidation inhibiting effect, but at a non-cytotoxic concentration of Resv, no enhancement of the GPX and SOD gene-promoting activities could be achieved.

Oxidative DNA damage has been suggested to be involved in the initiation and promotion step of carcinogenesis. Regarding tumour initiation, it should be mentioned that reactive oxygen species can directly induce single- or double-stranded DNA breaks, purine, pyrimidine or deoxyribose modifications, and DNA cross-links, thereby leading to mutations. Tumour promotion signal transduction pathways, including those in which AP-1 and NFkB are involved, are known to be activated by reactive oxygen species. AP-1 activation leads to increased cell proliferation, while NFkB is known to promote cell survival at least in part by inhibiting the apoptotic pathway. Based on their antioxidative properties, one would expect polyphenols to be able to inhibit the initiation and/or promotion of carcinogenesis. In this context, various recent studies have documented this to be the case. For example, Vásquez-Garzón et al. have shown that quercetin was
able to inhibit lipid peroxidation, to enhance the concentration of reduced glutathione and to increase the GPx, SOD and catalase activities in the liver of rats treated with diethylthiuroxamine, and in parallel to inhibit the development of neoplastic foci in the liver of these animals. Furthermore, the anti-tumor promoting effects of polyphenols present in a grape extract and black tea have been documented. Whether this is also the case of Vineatrol 30 is under investigation at the present time. This is particularly interesting if one takes into account that, on the one hand, Vineatrol 30 shows antioxidative capacity (this study) and, on the other hand, it exerts a direct antiproliferative effect on human cancer cells. 10,11

In summary, the results presented in this study clearly show that the Vineatrol 30 grapevine-shoot extract acted as a free radical scavenger and potent antioxidant at non-cytotoxic concentrations. Moreover, it was able to enhance the GPx and SOD gene-promoting activities, an effect that could not be achieved with Resv alone.

References