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Potent Inhibitory Action of Red Wine Polyphenols on Human Breast Cancer Cells

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Abstract Breast cancer (one of the most common malignancy in Western societies), as well as esophagus, stomach, lung, bladder, and prostate cancer, depend on environmental factors and diet for growth and evolution. Dietary micronutriments have been proposed as effective inhibitory agents for cancer initiation, progression, and incidence. Among them, polyphenols, present in different foods and beverages, have retained attention in recent years. Red wine is a rich source of polyphenols, and their antioxidant and tumor arresting effects have been demonstrated in different in vitro and in vivo systems. In the present study, we have measured the antiproliferative effect of red wine concentrate, its total polyphenolic pool, and purified catechin, epicatechin, quercetin, and resveratrol, which account for more than 70% of the total polyphenols in red wine, on the proliferation of hormone sensitive (MCF7, T47D) and resistant (MDA-MB-231) breast cancer cell lines. Our results indicate that polyphenols, at the picomolar or the nanomolar range, decrease cell proliferation in a dose- and a time-dependant manner. In hormone sensitive cell lines, a specific interaction of each polyphenol with steroid receptors was observed, with IC_{50}s lower than previously described. Interaction of polyphenols with steroid receptors cannot fully explain their inhibitory effect on cell proliferation. In addition, discrete antioxidant action on each cell line was detected under the same concentrations, both by modifying the toxic effect of H_2O_2, and the production of reactive oxygen species (ROS), after phorbol ester stimulation. Our results suggest that low concentrations of polyphenols, and consecutively, consumption of wine, or other polyphenol-rich foods and beverages, could have a beneficial antiproliferative effect on breast cancer cell growth. J. Cell. Biochem. 78:429–441, 2000. © 2000 Wiley-Liss, Inc.

Key words: polyphenols; wine; cancer cells (breast); cell proliferation; antioxidant

Breast cancer is one of the most common malignancies in Western societies. Its induction involves a multistep process, initiated with DNA damage, and followed by the alteration of different signaling pathways. Usually, at initial stages, breast cancer is hormone dependent, relaying on natural steroids. In later stages however, hormone independent growth is observed. Risk factors for this disease include age, race, ethnicity, hormones, and finally dietary factors [Gradishar and Morrow, 1996; Harris et al., 1997]. Epidemiological and animal studies have shown that different microchemicals present in the diet could be effective agents for the prevention of cancer incidence and mortality [Ames et al., 1995; Boone et al., 1990; Boone and Wattenberg, 1994; Kelloff et al., 1994; Morse et al., 1997; Wattenberg, 1992]. Measuring the effects of such agents has now become a major area of experimental cancer research. Among them, phenolic compounds are receiving increasing attention [Ames et al., 1995; Kelloff et al., 1994; Morse et al., 1997; Wattenberg, 1992].

Polyphenols belong to a heterogeneous class of compounds, found in plant sources, with a great variety of effects. They are potent antioxidant agents, interfering with the oxidative/

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antioxidative potential of the cell [Cheng, 1989; Fauconneau et al., 1997], or acting as free radical scavengers [Fauconneau et al., 1997; Kuo, 1997]. Inhibition of phosphodiesterase with increase of cyclic nucleotide concentrations, and inhibition of several protein kinases, involved in cell signaling [see Soleas et al., 1997, for a recent review] have been proposed as possible mechanisms of action. Polyphenols are absorbed from the upper gastrointestinal tract [Serafini et al., 1998], after wine ingestion. They are distributed in the body, showing an increased affinity for the heart, liver, and kidney, but chronic ingestion is necessary to obtain bioeffective concentrations [Bertelli et al., 1996a,b,c, 1998].

Grapes and wine contain a great amount of polyphenols, ranging from 12.6–22.4 mmol/L [Miller and Rice-Evans, 1995]. The grape cultivar, enological techniques, and climatic or microclimatic factors influence its chemical composition and its polyphenolic content [Miller and Rice-Evans, 1995]. Wine polyphenols include phenolic acids (p-coumaric, cinnamic, caffeic, gentisic, ferulic, and vanillic acid), trihydroxy-stilbenes (resveratrol and polydatin), and flavonoids (catechin, epicatechin, and quercetin). They are synthesized through a common pathway from phenylalanine, which requires the activation of a specific enzymatic system, including polyphenol oxidase, the rate limiting enzyme for their synthesis [Almeida and Nogueira, 1995]. Polymeric aggregation gives rise, in turn, to the viniferins (potent antifungal agents) and procyanidins (strong antioxidants that also inhibit platelet aggregation). Polyphenols, among other compounds, including vitamins, pigments, flavonoids, etc., possess antimutagenic properties [Bariliak and Isaeva, 1994], as well as blood glucose decreasing activity [Thompson et al., 1984].

The antioxidant effect of red wine and its major polyphenols have been demonstrated in many systems, from in vitro studies (human low density lipoprotein, liposomes, macrophages, cultured cells) to investigations in normal human subjects, although their effects remain controversial [Goldbohm et al., 1996; Graham, 1992; Hayatsu et al., 1992]. Several of these compounds (notably catechin, quercetin, and resveratrol) promote nitric oxide production by vascular endothelium, inhibit the synthesis of thromboxane in platelets and leucotriene in neutrofils, modulate the synthesis and secretion of lipoproteins in whole animal and human cell lines, arrest tumor growth and inhibit carcinogenesis in different experimental models [Soleas et al., 1997]. Usually, the reported effective concentrations of polyphenols range from 10^{-5} to 10^{-6} M, far above those achieved in the body fluids, after a moderate ingestion of wine (see Discussion).

In the present study, we have assayed the effect of low concentrations of red wine, as well as polyphenols isolated and purified from it, on the proliferation of three different human breast cancer cell lines, namely MCF7, T47D, and MDA-MB-231. Our results indicate that different polyphenols inhibit cell proliferation in a time- and dose-dependent manner, and interact with intracellular steroid receptors, although this later interaction cannot fully explain their antiproliferative action.

MATERIALS AND METHODS

Cell Lines and Culture Conditions

We have used three different human breast cancer cell lines: MCF7 and T47D which are hormone sensitive, while possessing a discrete pattern of estrogen and progesterone receptors, and MDA-MB-231, which is hormone independent for growth. MCF7 cells were purchased from DSMZ (Braunschweig, Germany), while T47D, and MDA-MB0231 cells were from the European Collection of Cell Cultures (Salisbury, UK). T47D cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS); MCF7 cells were cultured in DMEM/F12 medium, supplemented with 5 μg/ml insulin (Sigma, St Louis, MI) and 10% FBS, in a humidified atmosphere of 5% CO_2 in air. Finally, MDA-MB-231 cells were cultured in L-15 medium, supplemented with 2 mM glutamine, and 10% FBS, in the absence of CO_2. All culture media and serum were from Gibco BRL (Life Technologies, Paisley, UK).

Medium, supplemented with the different compounds, was changed every day. The serum batches used were assayed, prior to use, for the presence of polyphenol oxidase (seruloplasmin) and transferrin, by conventional nefelometric techniques, using a QM300 nefelometer, and commercial kits by Kallestad/Pasteur (Paris, France). There were no measurable levels of either substance found.

Wine and Polyphenols

Concentrated and desalcoholized wine, of the Cabernet Sauvignon cultivar, was obtained from the Centre des Recherches Agronomiques de Montpellier (France), by vacuum distillation, followed by evaporation. The wine concentrate (×5) was then clarified, pasteurized, and sealed in a wine bottle, conserved at 4°C, and used within the month it was opened. Wine total polyphenolic extract was produced in the same research center, by resin absorption of total polyphenols, followed by ethanol elution, and evaporation.

Catechin (+), epicatechin (−), and resveratrol (+) were prepared from the total polyphenolic extract of wine, by the Laboratoire de Pharmacognosie, Universite de Bordeaux 2 (Prof. J. Vercauteren), by semipreparative high-pressure liquid chromatography, using an RP18 column. Detection of each substance was made by UV-visible spectroscopy. Each substance was recrystalized, and its final purity, assayed by analytical HPLC, was >99%. This was also confirmed by proton nuclear magnetic resonance (NMR), at 500 MHz. Each polyphenol was conserved in a dark bottle, at −20°C, under nitrogen. Quercetin was purchased from Sigma chemicals (St. Louis, MI). The choice of the different molecules was made with the coordinator of the Scientific Council of the Association "Vin et Sante: Biologie et Pathologie Tumorale" (Aix en Provence, France).

Total wine polyphenolic extract and each polyphenol were initially diluted in absolute ethanol (10^{-2} M), and stored in dark bottles, at −20°C. From this initial source, working solutions were prepared, immediately before use, in Phosphate Buffered Saline (PBS). These solutions were introduced at the cell culture, at a volume of 10 μl, in a total volume of 1.0 ml of culture medium. Medium and the different polyphenols were replaced daily.

Cell Viability and Growth Assay

Cells were plated in 24-well plate, at an initial density of 2×10^4 cells, with 1.0 ml medium per well. After 24 h, in order to ensure uniform attachment of cells at the onset of the experiments (designated as day 0), polyphenols or wine were added to cultures. Cells were grown for a total of 6 days, with daily change of the medium and the different substances tested. Growth and viability of cells were measured by the tetrazolium salt assay [Mosmann, 1973]: Cells were incubated for 4 h at 37°C with the tetrazolium salt (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide), and metabolically active cells reduced the dye to purple formazan. Dark blue crystals were dissolved with propanol. The absorbance was measured at 570 nm and compared against a standard curve of known numbers of cells. At initial experiments, we have compared the tetrazolium salt assay with a direct measurement of cell number, in order to exclude any variation due to the presence of antioxidants in the medium or in the cell. No such effect was found, in any case. All experiments were performed a minimum of three times, in triplicate.

H_2O_2 Treatment

Cells were seeded in 24-well plates at an initial density of 150,000 cells/well. After 24 h, medium was replaced, and FBS was omitted. 24 h later, polyphenols (10^{-8} M) were added. After another 24 h, medium was replaced and different concentrations of H_2O_2 were added, varying from 0.05 to 5 mM. Cells were incubated for 3 h at 37°C, washed with PBS, and their viability was determined by the MTT method, as described above. Although preliminary experiments did not provide any evidence of an interference of the serum with H_2O_2, we have preferred to eliminate serum from all the experiments. Cell viability was not influenced, for the short periods of the experiment by the absence of serum.

Determination of Reactive Oxygen Species Generation by Flow Cytometry

Reactive Oxygen Species (ROS) production was assayed by flow cytometry, as described by Rothe and Valet [1994]. Briefly, 1 million cells, treated or not with 10^{-8} M wine polyphenols for 24 h, were removed from dishes, and loaded with dihydroxyrhodamine 123 (10 μl of a 100 μM solution in a total volume of 1 ml) and incubated for 7 min at room temperature. Thereafter, 10 μl of a solution of 10 μM phorbol-12 myristate, 13-acetate (PMA, Sigma) is added, incubated for another 5 min, and counted in a Coulter Epics flow cytometer (Coulter Electronics, Lutton, Bedfordshire, UK). In the presence of intracellular ROS, dihydroxyrhodamine 123 is transformed to

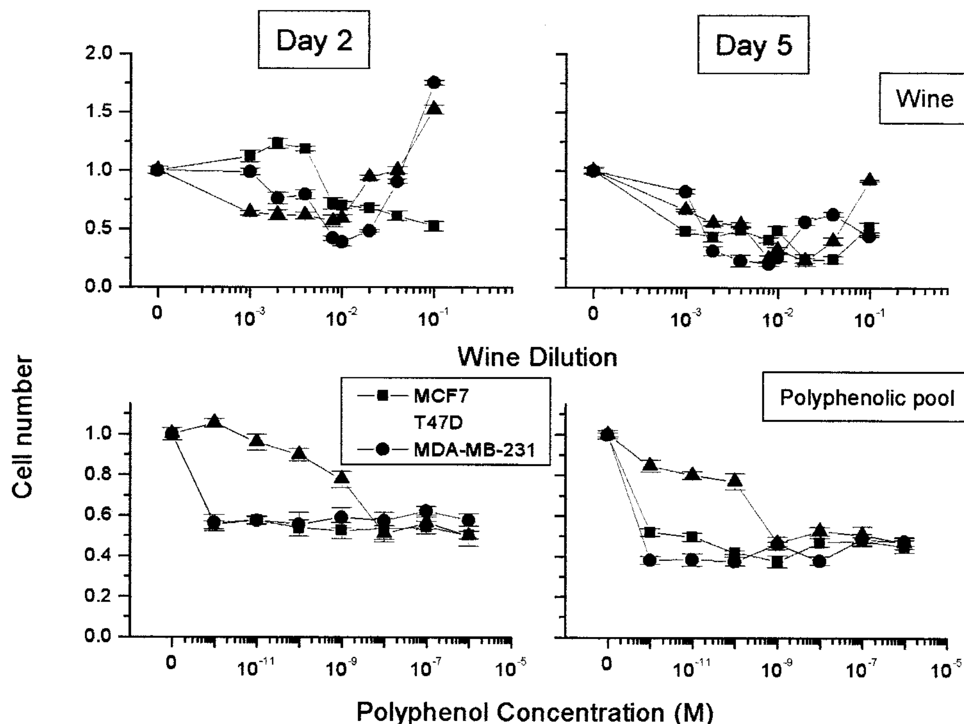

Fig. 1. Effect of desalcoholized wine and total polyphenols isolated from it, on cell proliferation of human breast cancer cell lines. Wine or polyphenols, were applied for 2 (one cell cycle) and 5 days (three cell cycles), and the cell number was compared to the control, that is cells cultured in the absence of the tested compound. At the onset of the experiment, 1×10^6 cells were seeded in six-well plates. Mean ± SE of a typical experiment in triplicate.

green-fluorescent rhodamine 123, trapped intracellularly. Measurements were repeated at determined time intervals for one hour, as indicated in the Results section.

Steroid Hormone Receptor Assay

One-hundred fifty $\times 10^3$ cells were seeded in 24-well culture dishes. After 24 h, medium was aspirated, and cells were washed with phosphate buffered saline. Binding was performed on whole cells, in serum-free phosphate buffered saline, in a total volume of 0.4 ml. For displacement binding of [^{3}H] estradiol (New England Nuclear, Zaventum, Belgium; Specific Activity 103 Ci/mmol) or [^{3}H] progesterone (Amersham, Buckinghabshire, UK; Specific Activity 96 Ci/mmol), were introduced at a concentration of 435 fmoles (~40,000 cpm) in 50 μl of PBS, together with varying concentrations of non-labelled estradiol (Sigma), ORG2058 (Amersham), or polyphenols, varying from 10^{-12} to 10^{-6} M. After overnight incubation at 4°C, under agitation, medium was aspirated, cells were washed twice with 1.0 ml ice-cold PBS, removed from plates with 1 ml 0.5 N NaOH, and mixed with 5 ml scintillation cocktail (SigmaFluor, Sigma). The bound radioactivity was counted in a scintillation counter (Tricarb, Series 4000, Packard), with 60% efficiency for Tritium. Binding was repeated at least three times (in duplicate). The results were analysed by the Origin (MicroCal Co., Northampton, MA) V. 5 package, using equations described by Munson and Rodbard [1980].

RESULTS

Cell Proliferation

Effect of wine concentrate on cell proliferation. Figure 1 presents the effect of desalcoholized wine on the proliferation of human breast cancer cell lines. After 2 days of incubation (one cell cycle) it is noted that wine produces a dose-dependant inhibitory effect on cell proliferation. T47D and MDA-MB-231 cells were most sensitive to this inhibitory effect, as compared to MCF7 cells. At concentrations lower or equal to 1/100 of wine, all cell lines were inhibited. At higher concentrations however (non-compatible nevertheless with moderate wine consumption, see Discussion), in two

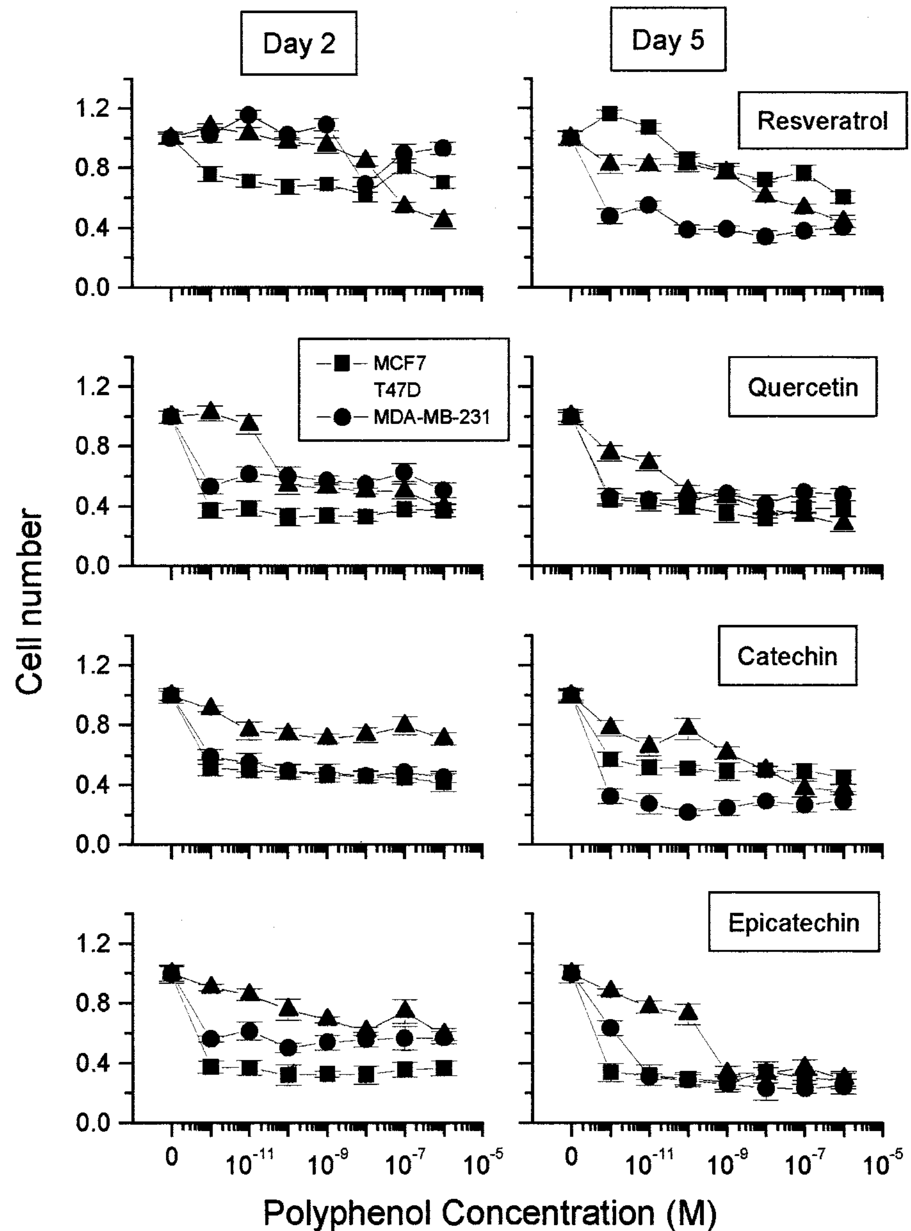

Fig. 2. Effect of polyphenols isolated from red wine, on cell proliferation of human breast cell lines. Each polyphenol, was applied for 2 (one cell cycle) and 5 days (three cell cycles), and the cell number was compared to a control, that is cells cultured in the absence of the tested compound. At the onset of the experiment, 1×10^6 cells were seeded in six-well plates. Mean ± SE of a typical experiment in triplicate.

of the cell lines (T47D and MDA-MB-231), this inhibition was inverted, and at high concentrations (1/10) a stimulation of cell proliferation was observed. After 5 days of incubation, the inhibitory effect was more pronounced, and the stimulatory effect was not observed. Under these conditions, the wine dilution, producing a half-maximal stimulation was in all cases 0.008 (1/125 dilution).

Polyphenolic pool and purified polyphenols. In contrast to wine, the polyphenolic pool purified from the same cultivar, showed a more potent inhibitory effect in the three cell lines (Fig. 1). It is interesting to note that the two hormone-sensitive cell lines (MCF7 and T47D) were more sensitive to the action of polyphenols, than the hormone resistant line MDA-MB-231, especially after short incubation times (IC_{50}s 0.14, 0.09, and 1.3 pM at day 2, and 0.16, 0.9, and 0.23 pM at day 5, respectively). This result indicates a possible implication of steroid hormone receptors in the action of polyphenols (see later results).

The effect of purified polyphenols is presented in Figure 2. All purified polyphenols produced a dose-dependent inhibition of cell

TABLE I. Inhibitory Concentrations and Maximal Inhibition of Red Wine Purified Polyphenols in Three Human Breast Cancer Cell Lines[a]

	Resveratrol		Quercetin		Catechin		Epicatechin	
Cell line	IC_{50} (pM)	Max. inhibition	IC_{50} (pM)	Max. inhibition	IC_{50} (pM)	Max. inhibition	IC_{50} (pM)	Max. inhibition
MCF7	13.7 ± 8.3	0.42	4.4 ± 5.6	0.39	0.4 ± 0.3	0.42	0.2 ± 1.2	0.46
T47D	0.1 ± 1.2	0.56	0.1 ± 0.2	0.69	0.1 ± 0.7	0.73	0.8 ± 0.2	0.75
MDA-MB-231	5.2 ± 9.1	0.30	2.4 ± 8.2	0.72	9.3 ± 8.9	0.61	7.4 ± 9.2	0.69

[a]Cells (MCF7, T47D, and MDA-MB-231) were incubated for a total of 6 days, in the presence of various concentrations of the indicated polyphenols, at concentrations varying from 10^{-12} to 10^{-6} M, with daily change of the medium and polyphenols. The inhibitory concentrations (IC_{50}) and the maximum inhibition were calculated by sigmoidal fitting of the data in the Origin V 5.0 microcomputer program. Mean of three different experiments performed in triplicate.

proliferation. The effect of polyphenols was more pronounced after three cell cycles (day 5), as compared to day 2. For almost all polyphenols tested (with the exception of resveratrol on MCF7 cells) hormone sensitive cell lines MCF7 and T47D, showed a greater inhibition than the hormone resistant MDA-MB-231, indicating a possible interaction of polyphenols with steroid receptors. IC_{50}s for each polyphenol tested are presented in Table I.

Interaction of Polyphenols With Steroid Hormone Binding Sites

Some phenols [Berthois et al., 1986] or polyphenols [Gehm et al., 1997; Komori et al., 1993; Kuo, 1997] were reported to be agonists of steroid receptors. In order to test this interaction, we have performed displacement experiments of ladiolabelled estradiol and progesterone with purified polyphenols, at concentrations compatible to those inhibiting cell proliferation. The results are presented in Figure 3, and Table II.

We have found a competition of polyphenols for steroid binding sites. In MCF7 cells, quercetin and catechin displaced estradiol from its receptors, at the picomolar range, while resveratrol and epicatechin interacted at the nanomolar range. At the same line, resveratrol and catechin interacted at the picomolar, while quercetin and epicatechin at the nanomolar range with progesterone receptors. In T47D cells, the observed interactions were less pronounced. Only resveratrol and epicatechin interacted with estrogen receptors (at the nanomolar and the picomolar range respectively) and resveratrol and quercetin with progesterone receptors. Finally, as expected, no steroid binding was observed at the MDA-MB-231 cell line.

Antioxidant Action

Wine polyphenols are major antioxidant agents, acting in plants as local antimicrobial agents. In the present study, we have assayed their antioxidant activity by measuring the protection of the different cell lines from exogenously applied oxidative agents, as well by the modification of reactive oxygen species produced by the cells, after phorbol ester stimulation.

Effect of polyphenols on the action of H_2O_2 on breast cancer cell lines. Figure 4 shows the modification of cell viability after the application of different concentrations of hydrogen peroxide. H_2O_2 was toxic at very low concentrations. The different cell lines tested could be distinguished in two groups, according to their susceptibility to the action of H_2O_2: T47D and MDA-MB-231 cells were very sensitive to the action of H_2O_2, which presents its cytotoxic effect at concentrations about 10^{-5} M. On the contrary, MCF7 cells are about 10 times more resistant to the action of H_2O_2.

The preincubation of cells with wine polyphenols (Fig. 4, Table III), produced different effects in each cell line. MDA-MB-231 cells did not show any significant change. On the contrary, all tested polyphenols produced a significantly higher resistance of T47D cells to the action of H_2O_2, while, in the MCF7 cell line, epicatechin had no effect, although all other substances increased the resistance of cells to the action of hydrogen peroxide by five times.

Production of ROS. Another way to measure the antioxidant activity of the different polyphenols, is the modification of reactive oxygen species production, after mitogen stimulation. In the present study PMA was used as stimulator. Our results are presented in Figure

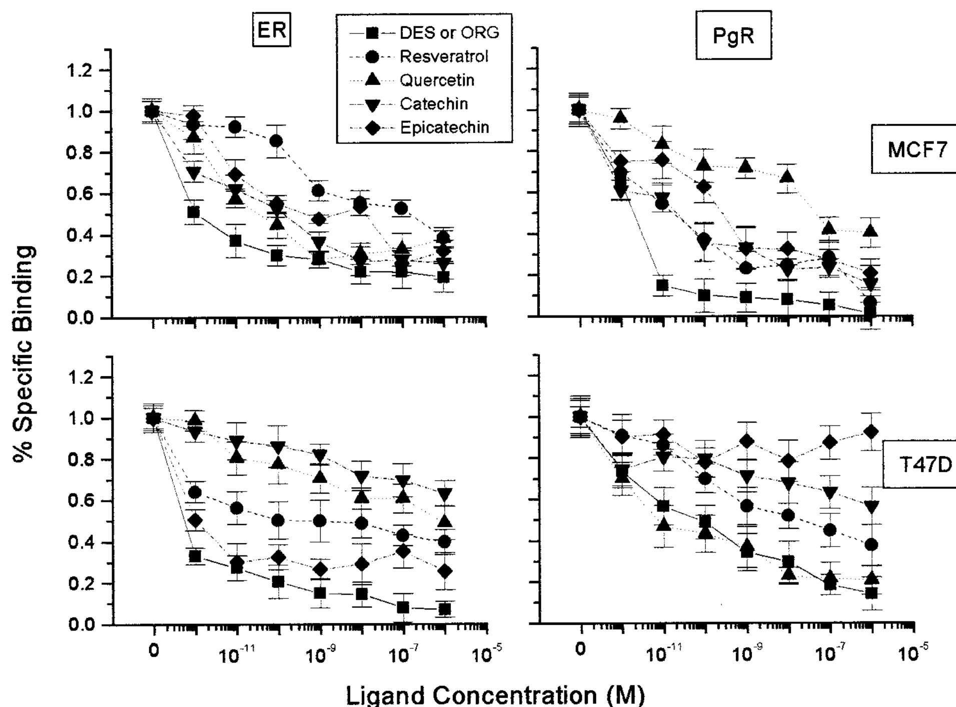

Fig. 3. Displacement of radiolabelled steroids ([^{3}H]estradiol for estrogen receptors (ER), and [^{3}H]progesterone for progesterone receptors (PgR)) by wine polyphenols in hormone sensitive breast and prostate cell lines. Mean ± SE of a typical experiment in triplicate.

TABLE II. Affinity of Polyphenols for Steroid Receptors in the Different Hormone Sensitive Breast Cancer Cell Lines[a]

	MCF7		T47D	
	ER (M)	PgR (M)	ER (M)	PgR (pM)
Homologous ligand (DES, ORG)	$1.19 \pm 0.54 \times 10^{-12}$	$1.84 \pm 3.7 \times 10^{-12}$	$0.76 \pm 0.40 \times 10^{-12}$	$23.5 \pm 11.4 \times 10^{-12}$
Resveratrol	$8.51 \pm 4.40 \times 10^{-9}$	$34.4 \pm 27.9 \times 10^{-12}$	$7.55 \pm 0.19 \times 10^{-9}$	$3.91 \pm 2.01 \times 10^{-9}$
Quercetin	$17.7 \pm 1.29 \times 10^{-12}$	$0.61 \pm 0.38 \times 10^{-9}$	$>10^{-7}$	$11.4 \pm 7.5 \times 10^{-12}$
Catechin	$22.2 \pm 1.18 \times 10^{-12}$	$38.3 \pm 33.3 \times 10^{-12}$	$>10^{-7}$	$>10^{-7}$
Epicatechin	$0.23 \pm 0.21 \times 10^{-9}$	$0.42 \pm 0.28 \times 10^{-9}$	$4.54 \pm 8.52 \times 10^{-12}$	$>10^{-7}$

[a]IC_{50}s were calculated from three different displacement experiments, by sigmoidal fitting.

5. The three cell lines showed different profiles, both under basal conditions and after preincubation with polyphenols: MDA-MB-231 cells showed a small ROS production, while polyphenols did not have any effect. In the two other cell lines, PMA produced a very high increase of ROS-positive cells, attaining 90% after 1 h. Nevertheless, in these two cell lines, wine polyphenols produced different effects: A very potent inhibition of ROS production was found in T47D cells, while an increase of ROS production was observed in the MCF7 cell line. In this later case, resveratrol and quercetin were the most potent agents.

DISCUSSION

Different epidemiological and animal studies suggest that micronutrients, present in food, could have antioxidant and/or antimitotic activities, interfering with cancer initiation, progression, or mortality [Ames et al., 1995; Boone et al., 1990; Boone and Wattenberg, 1994; Kelloff et al., 1994; Morse et al., 1997; Wattenberg, 1992]. In addition to vitamins C and E

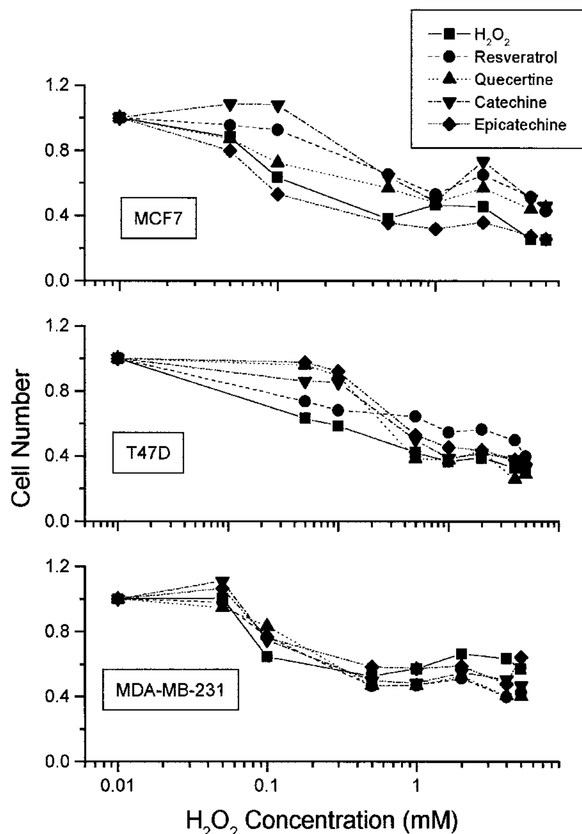

Fig. 4. Effect of H_2O_2 on cell viability. Effect of wine polyphenols. Cells were seeded in 24-well plates in an initial density of 150,000 cells/well. After 24 h, medium was replaced, and FBS was omitted. Twenty-Four h later, the different polyphenols, at a concentration of 10^{-8} M, were added in the culture medium. After another 24-h incubation, medium was replaced, and different concentrations of H_2O_2 were added, varying from 0.05 to 5 mM. Mean of a typical experiment performed in triplicate.

[Dabrosin and Ollinger, 1998], different agents were reported to possess an antioxidant activity, including melatonin [Baldwin and Barrett, 1998], tamoxifen [Custodio et al., 1994; Lim et al., 1992], estradiol, and progesterone [Dabrosin and Ollinger, 1998; Schor et al., 1999; Sipe et al., 1994]. Polyphenols, a constituent of different classes of foods and beverages, were thoroughly tested during the last years [Ames et al., 1995; Clifford et al., 1996; Conney et al., 1992; Huang et al., 1992, 1997; Kan et al., 1996; Katiyar et al., 1993; Kelloff et al., 1994; Morse et al., 1997; Wattenberg, 1992]. However, an increased consumption of polyphenol-rich foods and beverages is not actually established as a cancer-protective factor, and controversial results have been reported [Goldbohm et al., 1996; Graham, 1992; Hayatsu et al., 1992].

Many natural products are sources of polyphenols, including avocado, banana, apple, pear, peach, potato, eggplant, different mushrooms, palm, tomato, coffee, tea, and grapes. Wine—especially red wine—is a rich source of polyphenols [Miller and Rice-Evans, 1995], including phenolic acids (p-coumaric, cinnamic, caffeic, gentisic, ferulic, and vanillic acid), trihydroxy-stilbenes (resveratrol and polydatin), and flavonoids (catechin, epicatechin, and quercetin). From all these polyphenols, the trihydroxy-stilbene resveratrol has been reported to act as an antioxidant, a platelet aggregation inhibitor, an antimutagenic or antiproliferative agent, and a factor decreasing the risk of cardiovascular diseases [Belguendouz et al., 1998, 1997; Bertelli et al., 1996a,b,c, 1998; Celotti et al., 1996; Constant, 1997; Fauconneau et al., 1997; Fitzpatrick et al., 1993; Fontecave et al., 1998; Gehm et al., 1997; Goldberg, 1996; Kawada et al., 1998; Mgbonyebi et al., 1998; Miller and Rice-Evans, 1995; Pace-Asciak et al., 1995; Ragazzi et al., 1988; Shin et al., 1998; Soleas et al., 1997; Turrens et al., 1997; Uenobe et al., 1997]. Nevertheless, micromolar concentrations of resveratrol were needed to produce the above actions and, as was stated by Miller and Rice-Evans [1995], it is doubtful whether efficient concentrations could be achieved after ingestion of moderate quantities of wine. On the other hand, red wine contains, in addition to resveratrol, high concentrations of catechin and epicatechin, and appreciable concentrations of quercetin, at relative amounts 160, 70, and nine times greater than resveratrol [Miller and Rice-Evans, 1995]. Therefore, it seemed interesting to test the antiproliferative activity of these four compounds (catechin, epicatechin, quercetin, and resveratrol) on the proliferation of different breast cancer cell lines, at concentrations obtained after a moderate wine ingestion. Desalcoholized wine, and the total polyphenolic pool obtained from it, were also assayed for comparison.

Our results, presented in Figures 1 and 2, show a direct effect of wine on cell proliferation. Wine concentrate, on the other hand, had inhibitory or stimulatory effects, depending on the concentration used and the time of incubation, indicating that other substances, different from polyphenols could be involved in its stimulatory action, as the total polyphenolic pool,

TABLE III. Effect of Wine Polyphenols on the IC_{50} of H_2O_2 in Different Breast Cancer Cell Lines[a]

	0	Resveratrol	Quercetin	Catechin	Epicatechin
MCF7	0.21	1.29	0.59	1.60	0.11
T47D	0.060	0.33	0.24	0.27	0.41
MDA-MB-231	0.089	0.094	0.108	0.098	0.098

[a]Table presents IC_{50}s in the absence (0) or the presence of 10^{-8} M of the indicated polyphenols. IC_{50}s are in mM.

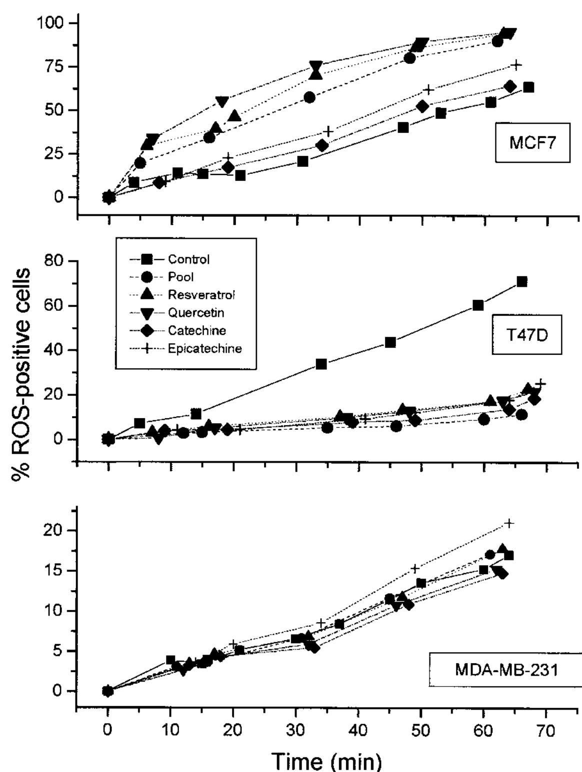

Fig. 5. Effect of wine polyphenols on the production of reactive oxygen species. One million of cells were treated or not with 10^{-8} M wine polyphenols or the total polyphenolic pool of red wine, as described in the Material and Methods section, for 24 h. Then, they were removed from dishes, and loaded with dihydroxyrhodamine 123 (10 μl of a 100 μM solution in a total volume of 1 ml) and incubated for 7 min at room temperature. Thereafter, 10 μl of a solution of 10 μM PMA is added, incubated for another 5 min, and counted in a Coulter Epics flow cytometer. Results of a typical experiment.

isolated from it, did not show this stimulatory action. On the other hand, purified polyphenols (Fig. 2) present a different effect on each cell line studied. In general, catechin and resveratrol were the most active antiproliferative agent in T47D cells, quercetin was more potent in MDA-MB-231, while resveratrol had an equal activity in T47D and MDA-MB-231 cells and epicatechin was equally potent in all three cell lines assayed. Furthermore, a discrete time effect was observed both for wine concentrate and the polyphenols used, confirming in vitro the in vivo epidemiological result that chronic ingestion of polyphenol-rich substances is necessary to obtain effective concentrations [Bertelli et al., 1996a, b, c, 1998]. As known, FBS contains a low amount of steroids. We have used this, instead of stripped serum, in order to mimic normal conditions, in humans, in which low concentrations of steroids can also be found in serum. Our results provide evidence that wine polyphenols can diminish cell growth, even in the presence of low concentrations of other stimulatory molecules, such as steroids, indicating a possible action of these agents, and therefore wine, under "physiological" conditions.

The mode of action of polyphenols is not well established (see the Introduction). Resveratrol, and perhaps other isoflavones, are agonists of the estrogen receptor [Gehm et al., 1997; Kuo, 1997], at concentrations of about three to 10 micromolar. It was further reported that a possible anticancer action of green tea polyphenols might be a decreased estrogen binding to their cognitive receptors [Komori et al., 1993]. In the present study, we have measured the interaction of wine polyphenols with steroid receptors, in the two hormone-sensitive cell lines, MCF7 and T47D. Our results are presented in Figure 3 and Table II. As shown, different polyphenols could interact with steroid receptors. It is interesting to note, that, in some cases, the observed IC_{50} for this interaction is at the low picomolar range, and that, usually, polyphenols, different from resveratrol, interact with a greater affinity with estrogen receptors. It is plausible therefore to consider, some of these compounds, as potent phytoestrogens. Nevertheless, the polyphenols tested, present a different affinity spectrum on MCF7 and T47D cells, both hormone sensitive, indicating that, possibly, other intracellular elements might be

involved, modifying their interaction with steroid receptors. This differential action requires further research. Nevertheless, as both hormone sensitive and resistant cell lines are inhibited by polyphenols, and different affinities for steroid receptors and IC_{50}s for cell inhibition were found, this interaction might not be the sole mechanism of the polyphenol inhibitory effect of cell proliferation. Indeed a direct antiproliferative effect of resveratrol on MCF7 and MCA cells was reported [Mgbonyebi et al., 1998], independent of estrogen receptor status, while the cancer chemopreventive activity of resveratrol [Jang et al., 1997], might be mediated either by estrogen receptors [Gehm et al., 1997; Kuo, 1997], or being estrogen-receptor independent [Mgbonyebi et al., 1998]. Perhaps, this effect could be due to the interruption of different signal transduction pathways [Mgbonyebi et al., 1998], or actin synthesis [Kawada et al., 1998]. Furthermore, it was equally shown that different polyphenols inhibit cell proliferation by increasing cells arrested at the G_2/M phase of the cell cycle [Okabe et al., 1997], a result shared by taxenes and opioid drugs [Panagiotou et al., 1999].

Our group has also studied the effect of polyphenols on the proliferation of other human cancer cell lines, deriving from different tissues. We have found that, in the prostate, wine polyphenols modify cell proliferation through a androgen receptor-independent mechanism, involving a decrease of NO production [Kampa et al., 2000]. This result, as well as the growth inhibition observed in MDA-MB 231 cells, indicate that the steroid receptor mediated cell growth inhibition, as presented here, might not be a general mechanism, but rather an element of polyphenol action. Other possible mechanisms of action might involve disruption of signaling pathways and modulation of nuclear factors [Chung et al., 1999; Dong et al., 1996; Draczynska-Lusiak et al., 1998; Lin et al., 1999]. Finally, it was reported that aryl hydrocarbon (aH) receptors could present a possible (although at higher concentrations) target of some dietary polyphenols, such as quercetin isomers [Johansson et al., 1982]. Furthermore, this intracellular receptor, presenting similarities with the steroid receptor, could be coupled, at the nuclear level, sharing some common responsive elements, as both estrogen and phenols can exert some actions, independently from their interaction with steroid receptors. These effects could possibly be mediated by an aH interaction of these agents [Moosmann and Behl, 1999]. Finally, reactive oxygen species are also implied in the activity of the aH receptor mediated cancer cell growth inhibition [Giardina et al., 1999]. As antioxidant polyphenols, modify the production of ROS (see Fig. 5) this interaction (mediated by TNF-alpha and NF-κB) could be a possible mechanism of action of polyphenols. This is currently under investigation.

The antioxidant effect of polyphenols is presented in Figures 4 and 5. It is noted that MCF7 cells, which were reported to produce high amounts of H_2O_2 [Szatrowski and Nathan, 1991], were the most resistant to the action of the oxidative agent (see also Table III). Only in hormone-sensitive cell lines, polyphenols antagonized the activity of H_2O_2, indicating that hormone sensitivity might possibly be involved in this antioxidant action. On the other hand, the same two cell lines (MCF7 and T47D) produced high amounts of ROS after phorbol ester stimulation (Fig. 5), while MDA-MB 231 cells produced five times less. In this case too, the effects of polyphenols was different. T47D cells decreased the production of ROS by about 80%, under polyphenols. In MCF7 cells, resveratrol and quercetin increased this production, while catechin and epicatechin had no effect. Finally in MDA-MB 231 cells, no significant action of polyphenols was observed. This difference could be attributed to different factors: 1. To the different constitutive production of ROS by the different cell lines [Szatrowski and Nathan, 1991]; 2. To the different hormone receptor spectrum of the cells, taking into consideration the reported interaction of ROS with these molecules [Hayashi et al., 1997; Liang et al., 1998; Townsend et al., 1991]; 3. To the fact that the method used for the assay of ROS includes equally the detection of nitric oxide [Rothe and Valet, 1994], which could be modified equally by antioxidant polyphenols, as indicated by results from our group in other tissues [Kampa et al., 2000].

Different wines have variable concentrations of polyphenols. In general, red wines have six to seven times more polyphenols, as compared to white ones [Sato et al., 1997; Serafini et al., 1998]. Taken into consideration the concentration of resveratrol or quercetin in red wines (5.4 and 302 μM, respectively) and the volume

of the interstitial fluid (~40 L), after the ingestion of half a liter of wine, the circulating resveratrol concentration will be about 70 and 3,800 nM, considering that no metabolism or excretion of the substance will occur. On the other hand, the existence in the serum of polyphenol oxidase (seruloplasmin), at concentrations varying between 23 and 61 mg/dl, depending on age and sex (results from our laboratory), makes more than probable the partial metabolism of polyphenols. Therefore, a possible effect of these substances, after ingestion of a moderate quantity of wine, must be detected at low concentrations, about the nanomolar or even the picomolar range, as found in the present study, indicating that moderate wine ingestion, or consumption of other foods and beverages, rich in antioxidant phenols, might have a protective effect in breast cancer.

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