

Do wine polyphenols modulate p53 gene expression in human cancer cell lines?

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Abstract

Background: The p53 gene is an established tumor suppressor and an inducer of apoptosis. We here attempt to determine whether the putative anticarcinogenic properties attributed to red wine and its polyphenolic constituents depend, at least in part, upon their ability to modulate p53 expression in cancer cells.

Methods: Three human breast cancer cell lines (MCF-7, T47D; MDA-MB-486) and one human colon cancer cell line [Colo 320 HSR (+)] were treated for 24-h with each of four polyphenols [quercetin; (+)-catechin, *trans*-resveratrol; caffeic acid] at concentrations ranging from 10⁻⁷ M to 10⁻⁴ M, after which, p53 concentrations were measured in cell lysates by a time-resolved fluorescence immunoassay.

Results: None of the polyphenols tested affected p53 expression in the breast cancer cell lines T-47D and MDA-MB-486. p53 content of MCF-7 breast cancer cells (wild-type) was increased by caffeic acid, decreased by resveratrol, and showed a twofold increase with catechin, that reached borderline statistical significance; however, none of these effects were dose-responsive. Colo 320 HSR (+) cells (with a mutant p53 gene) had lower p53 content upon stimulation, reaching borderline statistical significance, but without being dose-responsive, in the presence of caffeic acid and resveratrol. Apart from toxicity at 10⁻⁴ M, quercetin had no effect upon these four cell lines.

Conclusions: The observed p53 concentration changes upon stimulation by polyphenols are relatively small, do not follow a uniform pattern in the four cell lines tested, and do not exhibit a dose-response effect. For these reasons, we speculate that the putative anticarcinogenic properties of wine polyphenols are unlikely to be mediated by modulation of p53 gene expression. © 2001 The Canadian Society of Clinical Chemists. All rights reserved.

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1. Introduction

Red wines contain a large array of polyphenolic constituents that have been shown to block carcinogenesis and to inhibit the growth of tumors in whole animals, or in cell culture by altering the activity of certain enzymes or the expression of specific genes (See Refs [1] and [2] for review). The p53 gene has been implicated in normal cell proliferation, cell cycle control, induction of apoptosis, DNA repair, and carcinogenesis [3]. Its product is a 375-amino acid nuclear phosphoprotein that controls transcrip-

tional activation of a series of other genes, that cumulatively lead to cell cycle arrest and apoptosis [4–6].

Mutations in the p53 gene are believed to be crucial for transition of cells from the normal to the malignant phenotype [5]. The mutant p53 protein, which appears necessary for maintenance of this phenotype has a longer half-life than the wild-type protein. Whereas the latter is usually undetectable in normal cells, the mutant protein is overexpressed by 5 to 100-fold in transformed cells and tumor cell lines [4–6]. Not all human tumors have a p53 mutation, but this gene is by far the most commonly mutated gene among all malignancies with a frequency of approximately 50% [5].

The present study was undertaken to test the hypothesis that the anticancer activities of red wine polyphenols may, at least in part, be attributable to their ability to modulate p53 gene expression. We selected one representative com-

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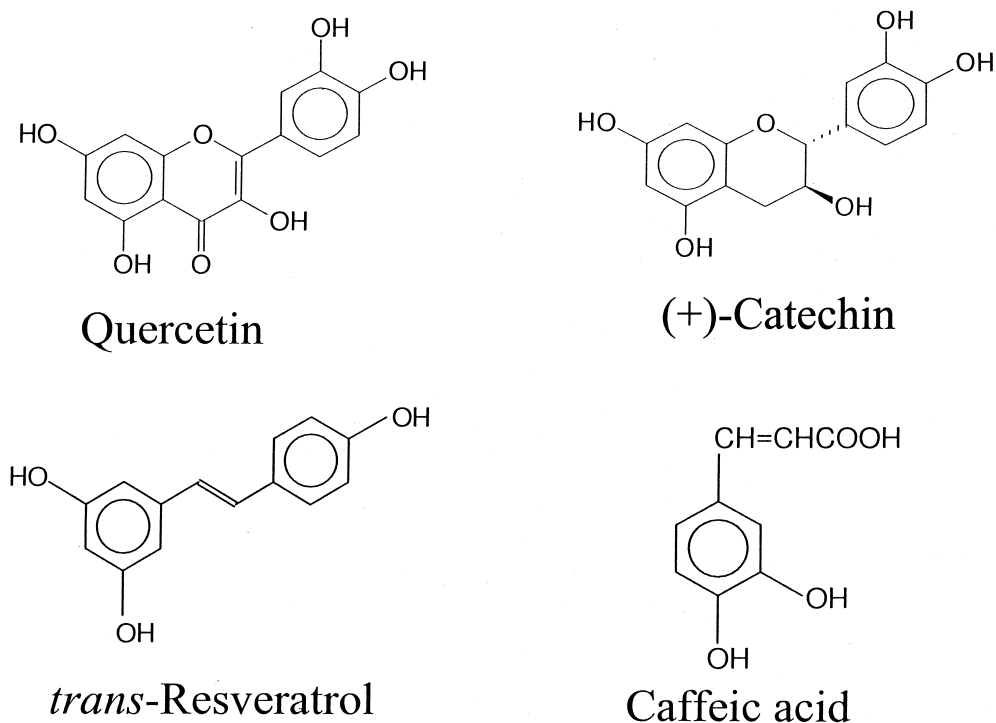


Fig. 1. Structures of polyphenols used in this investigation.

pound among the four major families of polyphenolic and phenolic substances in red wine: quercetin (a flavone); (+)-catechin (a flavan-3-ol); *trans*-resveratrol (a trihydroxystilbene); and caffeic acid (a hydroxycinnamate). Their structures are shown in Fig. 1.

2. Materials and methods

2.1. Phytochemicals

Phytochemicals, whose purity was stated to be >98%, were purchased from Sigma Chemical Co., St. Louis, MO, USA, as follows: quercetin (Cat. No. 17196–4); (+)-catechin (Cat. No. 86181–2); *trans*-resveratrol (Cat. No. R5010); caffeic acid (Cat. No. C0625). They were tested for purity with either high performance liquid chromatography with photodiode array detection (HPLC/PDA) or by gas chromatography/mass spectrometry [7,8].

2.2. Cell culture

The cell lines Colo 320 HSR (+)[colon carcinoma], MDA –MB-486 [breast carcinoma], T-47D [breast carcinoma] and MCF-7 [breast carcinoma] were obtained from the American Type Culture Collection (ATCC), Rockville, MD, USA. They were grown in culture media in 24-well plates as recommended by ATCC. At confluence, the number of cells per well approximated 0.2×10^6 . When cells were around 80% confluent, they were transferred to phe-

nol-free RPMI 1640 media, containing 10% charcoal-stripped fetal calf serum purchased from Hyclone Laboratories, Road Logan, UT, USA. Twenty-four hours later, fresh media were added and the cells were stimulated by adding polyphenols to final molar concentrations of 10^{-4} , 10^{-5} , 10^{-6} and 10^{-7} ($2 \mu\text{l}$ stock solutions in 2 ml media). Their p53 gene status (wild-type or mutant) was as follows: Colo 320 HSR (+), missense point mutation at codon 248; T-47D, missense point mutation at codon 194; MDA-MB-486, missense point mutation at codon 272; MCF-7, wild-type p53 [9]. For each phytochemical, 20 flasks were used as follows: quadruplicate measurements at the four stated concentrations plus a control (instead of phytochemicals, we added $2 \mu\text{l}$ of the stock solution diluent, ethanol).

The incubation times chosen were 6 h and 24 h post-stimulation, based on previous experience in which transcriptional activation became detectable within 2 to 3 h at the mRNA level and within 6 to 24 h at the protein level [10]. Cell viability was monitored by phase-contrast microscopy and trypan blue exclusion.

Following stimulation, the cells were detached by trypsin/EDTA treatment, transferred to tubes and washed twice with isotonic saline. They were then resuspended in isotonic saline and lysed as described [11].

2.3. p53 assay

A time-resolved immunofluorometric technique, described by Levesque *et al.* was used to measure the p53 content in cell lysates [11]. The limit of detection was 0.04

$\mu\text{g/L}$; the within-run imprecision (CV) was $<11\%$ and the between-run imprecision was $<15\%$ within the whole measurement range (0.04–75 $\mu\text{g/L}$). All p53 concentrations were corrected for the amount of total protein in cell lysates to compensate for cell numbers.

2.4. Total protein assay

Total protein was analyzed using the Pierce BCA protein assay reagent as recommended by the manufacturer (Pierce, Rockford, IL, USA).

2.5. Statistical analysis

A one-way analysis of variance (ANOVAR) was used to establish differences between the blank and the polyphenol concentrations tested. For those groups (cell line and polyphenol) that showed significant or borderline differences from the other treatment schedules (polyphenols) or controls within the same cell line by the Tukey-Kramer Multiple Comparisons Test (T-KMCT), two further procedures were performed: the method of Bartlett was used to test whether the differences among SDs for the columns within the same ANOVAR were significantly different; the method of Kolmogorov and Smirnov (KS) was applied to test whether the data were derived from populations that follow Gaussian distribution. Where the requirements of similar SDs and Gaussian distribution were not met, nonparametric ANOVAR (KW, Kruskal-Wallis test) was carried out and Dunn's Multiple Comparison Test (DMCT) was used to evaluate the significance of the observed differences. The theoretical basis for these procedures is described in Ref. 12 and they were carried out using the InStat 3 statistical package. In another approach, the data for all concentrations of a given polyphenol within a single cell line were pooled. Student's *t*-test (unpaired) was used to test whether the mean p53 content of the treated cells was significantly different from that of the controls (no polyphenol treatment).

3. Results

In no case did any of the polyphenols induce reduction or increase in p53 concentration in a dose-dependent manner, although in some experiments there was a moderate reduction or increase in p53 concentration over the entire range of polyphenol concentrations tested. Only in the 24-h cultures could p53 concentration be consistently and reliably measured; the 6-h data are therefore not presented.

3.1. T-47D and MDA-MD-486 cells

There was no significant effect with any of the polyphenols tested.

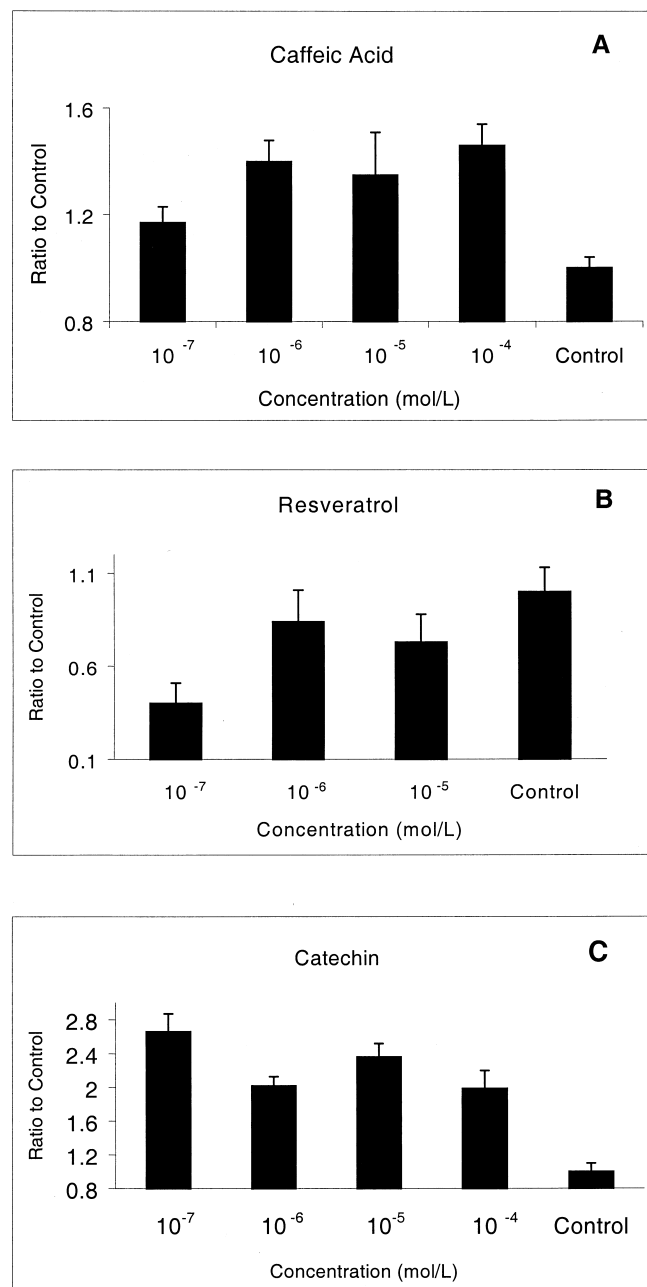


Fig. 2. Ratio of p53 content of MCF-7 cells grown in presence of various concentrations of caffeic acid (A), *trans*-resveratrol (B), and (+)-catechin (C) to that of controls grown in presence of solvent alone. The bars represent standard errors.

3.2. MCF-7 cells

With caffeic acid, the unpaired *t*-test gave a borderline value ($p = 0.058$) for the hypothesis that the p53 content in the presence of this polyphenol (all concentrations) was higher than in its absence (Fig. 2A). The distribution of data were Gaussian and the SDs were not significantly different. The one-way ANOVAR also strongly supported this hypothesis ($p = 0.0085$). With *trans*-resveratrol in this cell line, the mean for the controls was higher than that of all

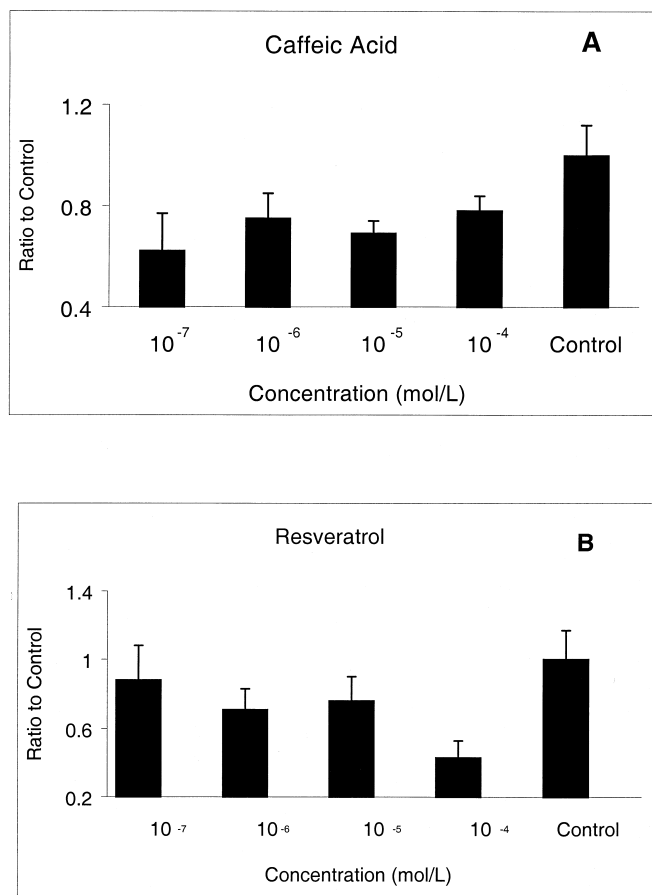


Fig. 3. Ratio of p53 content of Colo 320 HSR (+) cells grown in the presence of the various concentrations of caffeic acid (A) and *trans*-resveratrol (B) to that of controls grown in solvent alone. The bars represent standard errors.

treated cell cultures combined (Fig. 2B), generating a P-value of 0.045 for the unpaired *t*-test and <0.01 for the T-KMCT, following one-way ANOVA. The data were Gaussian and the SDs did not differ significantly.

In the case of (+)-catechin, the mean value for all treated cell cultures was higher than that of the controls (Fig. 2C). The P-value for the unpaired *t*-test was borderline ($p = 0.064$), but the T-KMCT, after one-way ANOVA was highly significant ($p < 0.001$). While the KS test revealed a Gaussian distribution of the data, Bartlett's test indicated a significant difference in SDs. These data suggest that (+) catechin increases p53 levels in MCF-7 cells.

3.3. Colo 320 HSR (+) cells

Both caffeic acid and *trans*-resveratrol appeared to lower the levels of p53 in this cell line (Fig. 3A and B). Following one-way ANOVA, the T-KMCT gave P-values of < 0.05 for caffeic acid and < 0.01 for *trans*-resveratrol, comparing all cells treated with these polyphenols against their respective controls. While the data were Gaussian, the SDs in both instances showed significant differences. Nonparametric

ANOVAR revealed significant differences, the KW test giving a P-value of 0.042 for *trans*-resveratrol. However, DMCT gave values of $p > 0.05$ for both caffeic acid and resveratrol, raising doubts about the statistical significance of these changes in this particular series of experiments. The other two polyphenols were without effect in this cell line.

4. Discussion

The notion that wine polyphenols can protect against cancer is widely held. Evidence favoring this view is based on epidemiologic surveys [13,14], tissue culture experiments [15,16], and whole animal studies [17,18]. One report described tumor remission in human terminal cancer patients treated with IV quercetin [19]. A number of plausible mechanisms have been proposed to account for these putative anticancer effects. Many of these polyphenols are powerful antioxidants and free-radical scavengers [20,21], as well as antimutagens [22,23]. Several have been shown to inhibit specific enzymes deemed to be important components of metabolic pathways associated with inflammation and cancer [24–27]. Thus, polyphenols may modulate various phases of the cell-cycle by interacting with key proteins involved in this process, including p53 [28–34].

The present investigation was undertaken to validate the hypothesis that the putative anticancer effects of wine polyphenols may be due, at least in part, to modulation of p53 gene expression. For this purpose, we employed human cancer cell-lines with characterized p53 gene status (wild-type or mutant). The choice of a representative compound from each of four polyphenol classes would allow us to evaluate the relative potency of these compounds or classes as p53 modulators.

We found no effects for any of these polyphenols with two breast cancer cell-lines, T47-D and MDA-MB-486. This finding is not in accordance with a report that quercetin down-regulates p53 in the related cell line MDA-MB-468 [28], but it should be pointed out that, more recently, quercetin was found to induce apoptosis in regenerating liver without affecting p53 synthesis [33,34].

With the MCF-7 breast cancer cell-line, which does not have a mutation in the p53 gene, and expresses only the wild-type protein, caffeic acid and (+)-catechin stimulation resulted in increased p53 concentration, while *trans*-resveratrol was associated with a decrease. Overall, the changes with all of these compounds were modest and none of these effects was dose-dependent. It was previously reported that flavonoids increase the cell content of wild-type p53, leading to enhanced rates of apoptosis [34]. We attempted to correlate changes in p53 expression with those of two tumor markers, carcinoembryonic antigen (CEA) and prostate-specific antigen (PSA), frequently found in human breast cancer [35], but their concentrations were undetectable in these cell-lines.

At 10^{-4} M, *trans*-resveratrol dramatically reduced p53

content to around 10% of the controls, but many cells showed severe morphologic damage under microscopy and were strongly stained by trypan blue, suggesting cytotoxic effects. The results were therefore not included in Fig. 2. Similar concentrations of quercetin also caused cell damage accompanied by marked reduction of p53 content in two of the cell lines utilized in this investigation (MDA-MB-486 and Colo 320 HSR (+)) but lower concentrations had little or no effect. Cytotoxicity does not appear to have been excluded in earlier reports describing reduced p53 content following exposure of cultured cancer cells to wine polyphenols. In Colo 320 HSR (+) cells, both caffeic acid and *trans*-resveratrol treatment were associated with lower p53 cell content. These effects were quite modest and were not dose-dependent. Their significance depended upon the choice of the statistical test employed.

In conclusion, our study shows little or no association between p53 protein levels and stimulation of cells by wine polyphenols. We believe that the previously observed effects of polyphenols on cell cycle and cell proliferation are mediated by mechanisms other than p53 gene modulation.

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