BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **250,** 53–58 (1998)
ARTICLE NO. RC989263

Resveratrol Arrests the Cell Division Cycle at S/G2 Phase Transition

Fulvio Della Ragione,[1] Valeria Cucciolla, Adriana Borriello, Valentina Della Pietra, Luigi Racioppi,* Giuliana Soldati,* Caterina Manna, Patrizia Galletti, and Vincenzo Zappia

*Institute of Biochemistry of Macromolecules, Medical School, Second University of Naples, Naples, Italy; and *Department of Cellular and Molecular Biology and Pathology, Medical School, University of Naples "Federico II," Naples, Italy*

Received July 27, 1998

Resveratrol (3,5,4′-trihydroxystilbene) is a naturally occurring phytoalexin, found in grapes and wine, which has been reported to exert a variety of important pharmacological effects. We have investigated the activity of resveratrol on proliferation and differentiation of the promyelocitic cell line HL-60. A concentration as low as 30 μM causes a complete arrest of proliferation and a rapid induction of differentiation towards a myelo-monocytic phenotype. Analyses by flow cytometry showed the absence of the G2/M peak and the accumulation of cells in G1 and S phases. Moreover, at the concentrations employed, a very low amount of apoptotic cells was evidenced. A detailed biochemical analysis demonstrated that the G1 phase of the cell division cycle engine was completely unmodified by resveratrol addition, thus indicating that the G1 → S transition occurs normally. Conversely, after only 24 h treatment, a significant increase of cyclins A and E could be observed along with the accumulation of cdc2 in the inactive phosphorylated form. These data demonstrate that resveratrol causes a complete and reversible cell cycle arrest at the S phase checkpoint.

Resveratrol (3,5,4′-trihydroxystilbene) is a naturally occurring molecule synthesized, similarly to other stilbenes, by several plants in response to pathogen attack, UV irradiation or exposure to ozone (1–3). The molecule, which is present at high level in grapes and wine, is classified as an anti-fungicide phytoalexin conferring disease resistance in the plant kingdom (1).

A large number of recent studies, carried out in cellular and animal models, demonstrated that resveratrol regulates many biological activities. The molecule has been reported to give protection against atherosclerosis exerting antioxidant activity (4), modulating the synthesis of haepatic apolipoprotein and lipids (5) and inhibiting platelet aggregation (6–8) and the production of proatherogenic ecosanoids by human platelets and neutrophils (6, 9). The down-regulation of prostaglandin and prostacyclin synthesis is probably due to the demonstrated inhibition of cyclooxygenase and hydroperoxidase activities (10). Since red wine represents the main resveratrol source, it has been proposed that some of the supposed beneficial effects of a moderate red wine intake on coronary degenerative disease(s) (the so-called French paradox) are due to the presence of this phytoalexin in the beverage (11, 12).

In addition to these findings, increasing interest has been paid to resveratrol due to the recent description of its cancer chemopreventive activity in assays analyzing the major stages (initiation, promotion and progression) of malignant transformation (10). Indeed, resveratrol is able to: (i) induce phase II drug-metabolizing enzymes; (ii) cause human promyelocytic leukemia cell differentiation, and (iii) inhibit the development of preneoplastic lesions in carcinogen-treated mouse mammary glands in culture and the tumorigenesis in a mouse skin cancer model (10). The antimutagenic activity of resveratrol, against the cancerogen 3-amino-1,4-dimethyl-5H-pyrido[4,3-β]indole, was also demonstrated by Ames assay using Salmonella typhimurium (13).

The molecular mechanism(s) of resveratrol action has not been well clarified. As above reported, the molecule is endowed with antioxidant properties and is able to inhibit efficaciously the hydroperoxidase activity of type 1 cyclooxygenase (10). Moreover, recent reports have demonstrated that this plant stilbene is able to inhibit ribonucleotide reductase (14) and DNA polymerase (15). Finally, it has been reported that resveratrol, like several other naturally occurring compounds,

[1] To whom correspondence should be addressed. Fax: +39-81-441688. E-mail: dellarag@cds.unina.it.

Abbreviations used: araC, arabinoside cytidine; cdk, cyclin-dependent kinase.

0006-291X/98 $25.00

mimics estradiol, and thus it might act as a phytoestrogen (16). However, no definite proof that these effects could explain the large variety of biological functions of the molecule has been obtained.

On the basis of the reported findings and of the relevance of resveratrol as a promising chemoprotective component of human diet, we investigated the effect of the molecule on cell proliferation and differentiation. In particular, we were interested in characterizing the precise mechanism of action of the compound on the cell division cycle engine. In the present report, we demonstrate for the first time that this phytoalexin inhibits cell proliferation hampering S → G2/M transition.

MATERIALS AND METHODS

Cells and cellular treatments. The HL-60 cells were obtained from American Type Culture Collection. The cell line was grown in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum and penicillin-streptomycin in a 5% CO_2 atmosphere at 37°C in a humidified incubator. Resveratrol (Sigma Chemical Company, St. Louis, MO) was resuspended in dimethyl sulfoxide at a concentration of 100 mM and stored at −80°C. The molecule was directly added to cell cultures at the indicated concentrations while untreated cells contained the solvent alone. Vitamin D3 (Sigma) was resuspended in absolute ethanol and stored at -20°C. The molecule was incubated with the promyelocitic cell line at a concentration of 1 μM in order to induce myelo-monocytic HL-60 cell differentiation. Arabinoside cytidine (AraC, Sigma) were dissolved in distilled water, sterilized by filtration and added to HL-60 cells at a concentration of 10 and 100 μM. This treatment was carried out to cause HL-60 cell apoptosis.

HT-29 cells were grown as described in 17. Sodium butyrate (Sigma) was dissolved in distilled water at a final concentration of 100 mM, sterilized by filtration, and stored at -20°C. The short fatty acid was added to HL-60 and HT-29 cells at 1 mM and 2 mM concentration respectively, in order to enhance $p21^{CIP1}$ protein expression.

Antibodies. Monoclonal antibodies to cyclin A, cyclin E, cdc2 were provided from Santa Cruz Biotechnologies, Santa Cruz, CA. Monoclonal antibodies to pRb and $p21^{CIP1}$ were from PharMingen, San Diego, CA while those to $p27^{Kip1}$ were from Transduction Laboratories, Lexington, UK. Polyclonal antibodies to cdk2, cdk4, cdk6, cyclin D1, cyclin D2, cyclin D3 were from Santa Cruz.

Immunochemical methods. Cell extracts were prepared as described in 17 and 18. 10–80 μg of cell extracts was analyzed by SDS–PAGE employing different percentage of acrylamide resolving gel, transferred to a nitrocellulose membrane and incubated with different antisera (18). The immunocomplexes were detected by the alkaline phosphatase method or by the enhanced chemoluminescent technique (Amersham, Bucks, U.K.) as described in 18.

Differentiation assays and flow cytometry analyses. The differentiation of HL-60 cells was evaluated by analyzing the nitroblue tetrazolium reduction and the nonspecific esterase activity. Nitroblue tetrazolium reduction activity was determined colorimetrically as described in ref. 19. Nonspecific acid esterase activity was assayed microscopically by employing a standard kit (Sigma) and following manufacturer's instruction. Flow cytometry analyses were carried out as described in 20. In these experiments, arabinoside cytidine was added to HL-60 cultures at the final concentration of 10 and 100 μM as a positive control of apoptotic process.

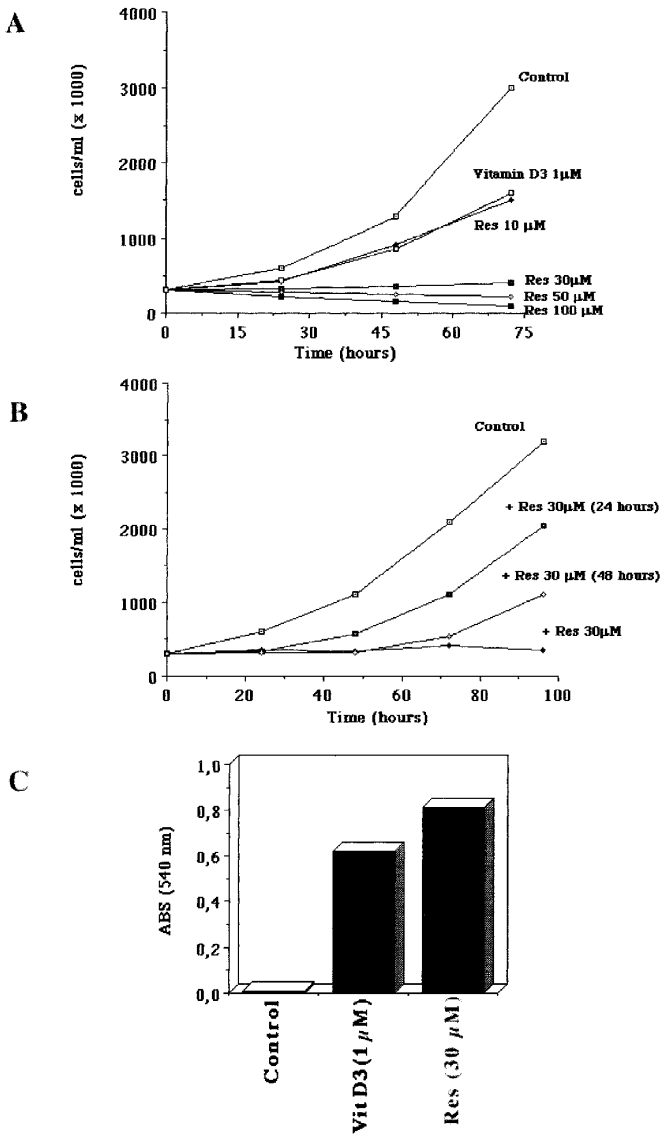

FIG. 1. Effect of resveratrol on HL-60 growth and differentiation. (A) HL-60 cells were plated at 300.000 cells/ml and incubated with or without different resveratrol amounts or with 1 μM vitamin D3. Cells were then counted daily. (B) HL-60 cells were treated with or without 30 μM resveratrol. After 24 (□) or 48 h (♦), resveratrol was removed and the cells were resuspended in fresh medium. Cells were then counted daily. (C) HL-60 cells were growth with or without the reported amount of resveratrol or vitamin D3. After 48 h, cells were collected and assayed for nitroblue tetrazolium reduction activity as reported in Ref. 19. The reported results are the media of three different experiments with less than 5% variation.

RESULTS AND DISCUSSION

Effect of resveratrol on growth and differentiation of HL-60 cells. When HL-60 cells were grown in the presence of different amounts of resveratrol, a remarkable inhibitory effect was observed (Fig. 1A). As low as 30 μM concentration resulted in a complete growth

inhibition, while at higher concentration (50 and 100 μM) the molecule appeared to be toxic, causing a decrease of cell number and a clear cellular fragmentation. From these experiments the estimated resveratrol I_{50} value was around 10 μM.

As shown in Fig. 1B, incubation of cells with the phytoalexin did not cause a definite commitment towards a non-proliferating state. Indeed, when the cells were incubated for 24 or 48 h with 30 μM resveratrol and then the drug was removed, a normal rate of proliferation was recovered. These results indicated that, under the conditions employed, the inhibition of growth was powerful but rapidly reversible, and that the molecule interferes, not irreversibly, with molecular process(es) required for cell proliferation.

Subsequently, we evaluated the differentiation activity of resveratrol. In these experiments we employed the expression of nitroblue tetrazolium reduction and of nonspecific acid esterase activities as markers of lineage commitment. Moreover, we used vitamin D3 as a positive control of differentiation induction. As shown in Fig. 1C, resveratrol addition caused a significant increase of nitroblue tetrazolium reduction which is a well known marker of differentiation of HL-60 towards the myelo-monocytic lineage. Interestingly, a similar result was obtained by vitamin D3 treatment. Moreover, an increase of cells showing nonspecific acid esterase activity was observed by microscopic analysis (data not shown).

In order to rule out the possibility that the inhibitory activity of resveratrol was related to the induction of apoptosis, we analyzed resveratrol-treated HL-60 by flow cytometry. The results (Figs. 2A and 2B) indicated that up to 30 μM concentration a very low percentage of programmed cell death could be detected (about 4%), which was superimposable to the value obtained with the untreated cells. At higher concentration, an increase of apoptosis could be observed. Interestingly, the flow cytometric analysis indicated the complete absence of G2/M peak and the accumulation of cells in G1/S phases.

In conclusion, resveratrol at concentrations comparable to those occurring in wine and grapes causes a complete arrest of HL-60 cell proliferation. This block was fully reversible, not due to the induction of apoptosis, and associated with a differentiation towards the myelo-monocytic phenotype.

Biochemical analysis of cell division cycle engine in resveratrol-treated HL-60 cells. The results obtained by flow cytometry prompted us to clarify the precise transition of cell division cycle hampered by resveratrol addition. As well known, the progression through the cell cycle is due to the timely regulated activation of serine/threonine cyclin-dependent kinases (cdks), a class of enzymes whose active form requires the binding between a catalytic subunit and an activating subunit, i.e., cyclin. Besides cyclin interaction, other regulatory events, including positive and negative phosphorylation and binding to cdk inhibitors, control cdk activity.

In a preliminary approach, we investigated whether the antioxidant molecule causes the induction of cdk inhibitors. Indeed, it has been recently demonstrated that other antioxidants (namely pyrrolidinedithiocarbamate and vitamin E) induce the increase of $p21^{CIP1}$ protein, a well-known cdk inhibitor (21). In addition, it has been firmly established that several molecules (including 1,25-dihydroxyvitamin D3, *trans*-retinoic acid and 12-O-tetradecanoylphorbol-13-acetate) induce HL-60 proliferation arrest and differentation by increasing the cellular content of two cdk inhibitors, namely $p21^{CIP1}$ and $p27^{Kip1}$ (22–24).

Analysis of cell extracts by immunoblotting demonstrated that the G1 phase cell division cycle engine (including cdk4, cdk6, cdk2, $p21^{CIP1}$, $p27^{Kip1}$, and cyclins D1, D2, and D3) was totally unmodified by resveratrol addition (Fig. 3 and data not shown). Moreover, we analyzed the phosphorylation degree of the retinoblastoma protein as a proof of cdk2 and cdk4/cdk6 activities. The amount of the hyperphosphorylated form of the retinoblastoma protein was identical in the treated and untreated cells (data not shown), thus confirming that resveratrol does not modify the G1-phase cdk activities. Therefore, on the basis of these results, we ruled out the occurrence of a G1 phase arrest.

Subsequently, we analyzed the content of cyclin E and cyclin A in HL-60 cells treated with 30 μM resveratrol. As shown in Fig. 3, a very high increase of these two cyclins was evidentiable after 2 days of treatment and was already clearly observable at 24 h after resveratrol addition. This result is consistent with the presence of a S $\rightarrow$ G2 block (25, 26).

To confirm this hypothesis, we analyzed the amount of cdc2 phosphorylation. Indeed, it has been recently demonstrated that the accumulation of the inactive tyrosine 15-phosphorylated (Y-15) cdc2 form is a definite evidence of a cell division cycle arrest preventing the entry in G2 and M phases (27, 28). As shown in Fig. 3, resveratrol treatment causes a high increase of nonfunctional Y-15 phosphorylated cdc2 form. This result, along with the increase of cylin A and E, clearly demonstrated that the treatment with the natural phytoalexin causes an arrest of the cell division cycle at the S phase checkpoint.

In summary, we have demonstrated by flow cytometry and detailed biochemical analyses that resveratrol blocks cell proliferation at the S/G2 boundary, thus causing the elongation of S phase and the activation of the differentiation program. This mechanism has been previously demonstrated for molecules hampering

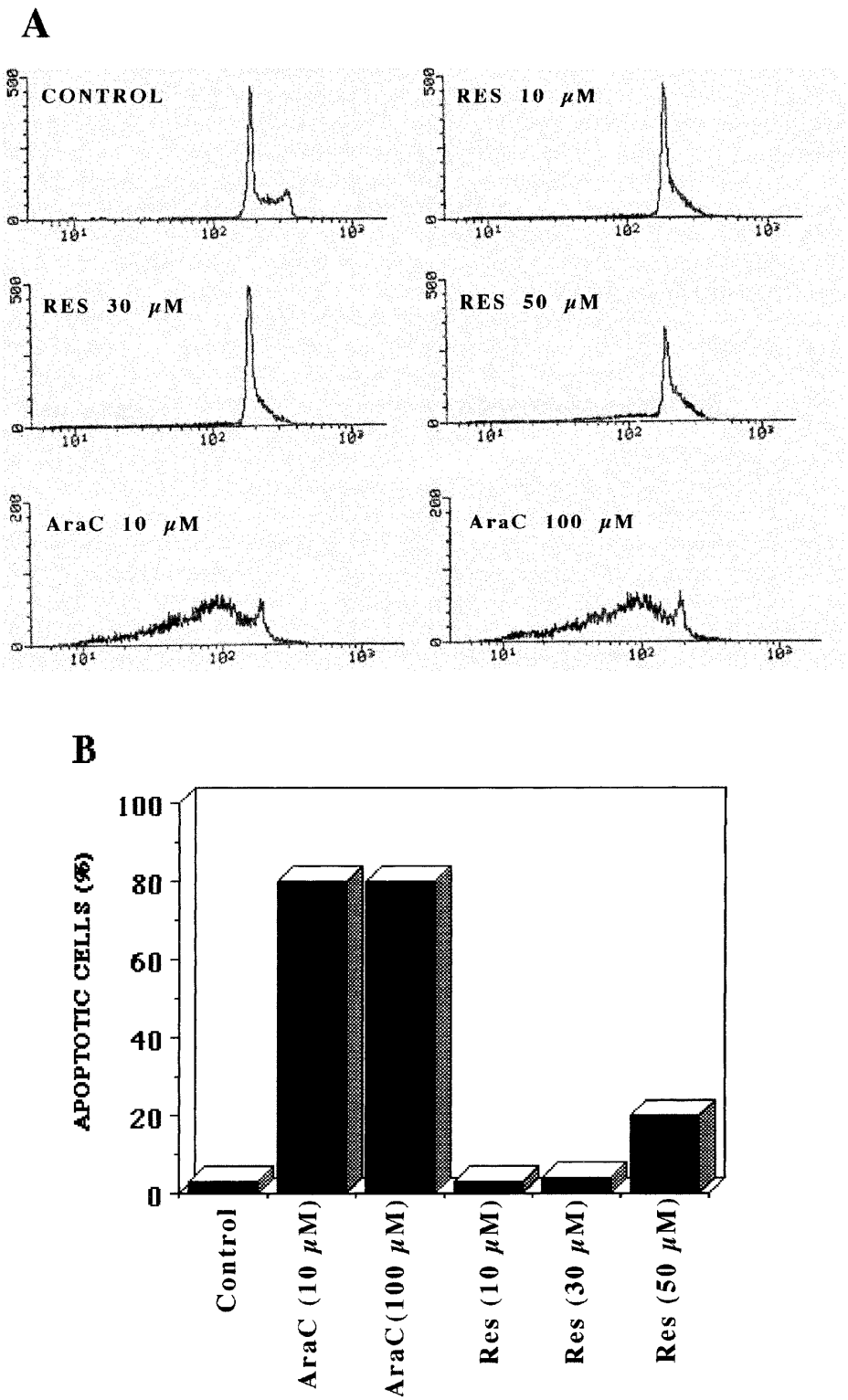

FIG. 2. Flow cytometric analysis of resveratrol-treated HL-60 cells. (A) HL-60 cells were grown for 48 h in the presence or absence of different amounts of resveratrol (Res 10, 30, and 50 μM) or arabinoside cytidine (Ara C 10 and 100 μM). Subsequently, cells were harvested and analyzed by flow cytometry as described in Ref. 20. (B) Percentage of apoptosis in HL-60 cells treated and analyzed as in A.

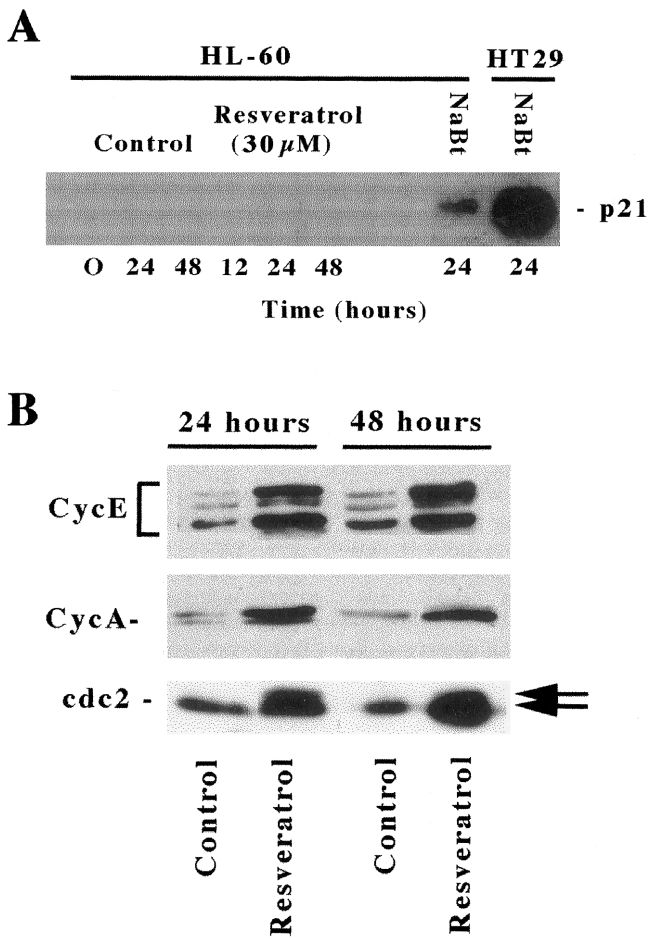

FIG. 3. Immunoblotting analysis of cell division cycle engine in resveratrol-treated HL-60 cells. (A) $p21^{CIP1}$ content of HL-60 treated with 30 μM resveratrol or 1 mM sodium butyrate (NaBt) and of HT-29 cells grown in 2 mM sodium butyrate-containing medium. About 80 μg protein were separated by denaturing polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and incubated with monoclonal antibodies to the cyclin-dependent kinase inhibitor. (B) Cyclin E, cyclin A and cdc2 analyses in resveratrol-treated HL-60 cells. 20 μg protein were used for cyclin immunoblotting while, in the cdc2 case, 10 μg protein were employed. The top arrow represents the Y-15 cdc2 phosphorylated form and the bottom arrow the unphosphorylated form.

DNA synthesis or inhibiting the topoisomerase activity (29, 30).

Recent reports suggest that resveratrol inhibits key enzymatic activities involved in DNA duplication, namely ribonucleotide reductase (14) and DNA polymerase (15). Therefore, we propose that the naturally occurring stilbene inhibits DNA synthesis thus preventing the activation of mechanisms which allow the overcoming of the S phase checkpoint. Elongation of S phase allows cell differentiation or, probably at high resveratrol concentration, the activation of apoptotic process.

In this scenario, the molecule is very attractive for the development of anticancer treatment as well as for inhibiting lymphocyte proliferation during immunosuppressive therapies.

ACKNOWLEDGMENTS

This work was supported in part by grants from the Italian Ministry of the University and Scientific Research (40 and 60%).

REFERENCES

1. Hain, R., Bieseler, B., Kindl, H., Schroder, G, and Stocker, R. (1990) *Plant Mol. Biol.* **15,** 325–335.
2. Soleas, G. J., Diamandis, E. P., and Goldberg, D. M. (1997) *Clin. Biochem.* **30,** 91–113.
3. Schubert, R., Fischer, R., Hain, R,. Schreier, P. H., Bahnweg, G., Ernst, D., and Sandermann, H. Jr. (1997) *Plant Mol. Biol.* **34,** 417–426.
4. Fauconneau, B., Waffo-Teguo, P., Huguet, F., Barrier, L., Decendit, A., and Merillon, J. M. (1997) *Life Sci.* **61,** 2103–2110.
5. Frankel, E. N., Waterhouse, A. L., and Kinsella, J. E. (1993) *Lancet* **341,** 1103–1104.
6. Pace-Asciak, C. R., Hahn, S., Diamandis, E. P., Soleas, G., and Goldberg, D. M. (1995) *Clin. Chim. Acta* **235,** 207–219.
7. Pace-Asciak, C. R., Rounova, O., Hahn, S. E., Diamandis, E. P., and Goldberg, D. M. (1996) *Clin. Chim. Acta* **246,** 163–182.
8. Bertelli, A. A., Giovannini, L., Bernini, W., Migliori, M., Fregoni, M., Bavaresco, L, and Bertelli, A. (1996) *Drugs Exp. Clin. Res.* **22,** 61–63.
9. Kimura, Y., Okuda, H., and Arichi, S. (1985) *Biochim. Biophys. Acta* **834,** 275–278.
10. Jang, M., Cai, L., Udeani, G. O., Slowing, K. V., Thomas, C. F., Beecher, C. W., Fong, H. H., Farnsworth, N. R., Kinghorn, A. D, Mehta, R. G., Moon, R. C., and Pezzuto, J. M. (1997) *Science* **275,** 218–220.
11. Sieman, E. H., and Creasy, L. L. (1992) *Am. J. Enol. Vitic.* **43,** 49–52.
12. Goldberg, D. M., Karamanchin, A., Yan, J., Soleas, G., Ng. E., Waterhouse, A. L., and Diamandis, E. P. (1995) *Am. J. Enol. Vitic.* **46,** 159–165.
13. Uenobe, F., Nakamura, S., and Miyazawa, M. (1997) *Mutat. Res.* **373,** 197–200.
14. Fontecave, M., Lepoivre, M., Elleingand, E., Gerez, C., and Guitter, O. (1998) *FEBS Lett.* **421,** 277–279.
15. Sun, N. J., Woo, S. H., Cassady, J. M., and Snapka, R. M. (1998) *J Nat. Prod.* **61,** 362–366.
16. Gehm, B. D., McAndrews, J. M., Chien, P. Y., and Jameson, J. L. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 14138–14143.
17. Della Ragione, F., Russo, G. L., Oliva, A., Mastropietro, A., Mancini, A., Borrelli, A., Casero, R. A., Iolascon, and Zappia, V. (1995) *Oncogene* **10,** 827–832.
18. Della Ragione, F., Russo, G. L., Oliva, A., Mercurio, C., Mastropietro, S., Della Pietra, V., and Zappia, V. (1996) *J. Biol. Chem.* **271,** 15942–15946.
19. Makishima, M., Kanatani, Y., Yamamoto-Yamaguchi, Y., and Honma, Y. (1996) *Blood* **87,** 3384–3394.
20. Nicoletti, I., Migliorati, G., Pagliacci, M. C., Grignai, F., and Riccardi, C. (1991) *J. Immunol. Methods* **139,** 271–279.
21. Chinery, R., Brockman, J. A., Peeler, M. O., Shyr, Y., Beauchamp, R. D., and Coffey, R. J. (1997) *Nature Med.* **3,** 1233–1241.

22. Steinman, R. A, Hoffman, B., Iro, A., Guillouf, C., Liebermann, D. A., and El-Houseini, M. E. (1994) *Oncogene* **9,** 3389–3396.
23. Jiang, H., Lin, J., Su, Z.-z., Collart, F. R., Huberman, E., and Fisher, P. B. (1994) *Oncogene* **9,** 3397–3406.
24. Hengst, L., and Reed, S. I. (1996) *Science* **271,** 1861–1864.
25. Pagano, M., Pepperkok, R., Verde, F., Ansorge, W., and Draetta, G. (1992) *EMBO J.* **11,** 961–971.
26. Rosenblatt, J. J., Gu, Y., and Morgan, D. O. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 2824–2828.
27. O'Connor, P. M., Ferris, D. K., Pagano, M., Draetta, G., Pines, J., Hunter, T., Longo, D. L., and Kohn, K. W. (1993) *J. Biol. Chem.* **268,** 9298–8308.
28. O'Connor, P. M., Ferris, D. K., Hoffmann, I., Jackman, J. Draetta, G., and Kohn, K. W. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 9480–9484.
29. Shao, R. G., Cao, C. X., Shimizu, T., O'Connor, P. M., Kohn, K. W., and Pommier, Y. (1997) *Cancer Res.* **57,** 4029–4035.
30. Nurse, P. (1997) *Cell* **91,** 865–867.