Original Article

Resveratrol Inhibits Proliferation of Primary Rat Hepatocytes in G0/G1 by Inhibiting DNA Synthesis

(resveratrol / epidermal growth factor / primary and transformed hepatocytes / proliferation inhibition / cell cycle inhibition)

J. A. RUBIOLO[1,2,] H. LÓPEZ-ALONSO[2], V. MARTÍN-VÁZQUEZ[2], N. M. FOL-RODRÍGUEZ[1], M. R. VIEYTES[1], L. M. BOTANA[2], F.V. VEGA[1]

[1]Departamento de Fisiología, [2]Departamento de Farmacología, Facultad de Veterinaria, Universidad de Santiago de Compostela, Lugo, Spain

Abstract. Resveratrol is a phytoalexin that has been shown to inhibit cell proliferation of several cancer cell lines. In some cases this inhibition was specific for the transformed cells when compared with normal cells of the same tissue. To test whether this was the case in rat hepatocytes, we exposed primary rat hepatocytes in culture and transformed rat hepatic cells to this compound and studied its effect on cell proliferation, measuring deoxy-bromouridine incorporation and total DNA. We also studied the effect of resveratrol on the cell cycle of normal and transformed rat hepatocytes. We observed that resveratrol inhibited proliferation in a dose-dependent manner in both cases, with no differential action in the transformed cells compared to the normal ones. This compound arrested the cell cycle in G0/G1 in primary hepatocytes, while it arrested the cell cycle in G2/M in transformed cells. Transformed hepatocytes showed accumulation of cells in the S phase of the cell cycle.

Introduction

Resveratrol (*trans*-3,5,4´-trihydroxystilbene) is a phytoalexin. Its synthesis in plants can be induced by microbial infections, UV light and exposure to ozone (Ignatowicz and Baer-Dubowska, 2001). Resveratrol is present in fruits, vegetables, and beverages including wine that are part of the human diet (Fremont, 2000). Several reports indicate that resveratrol inhibits proliferation of cancer cells including myeloid leukaemia cells (Estrov et al., 2003), epidermoid carcinoma A431 cells (Ahmad et al., 2001), colon carcinoma HT29 and SE480 cells (Delmas et al., 2002; Liang et al., 2003), hepatocarcinoma Fao and HepG2 cells, breast cancer MCF cells (Mgbonyebi et al., 1998) and prostate cancer LNCaP cells (Hsieh and Wu, 1999). In some cases, the resveratrol concentration needed for cell proliferation inhibition was found to be lower in cancer cells than in normal cells of the same tissue. In leukaemia cells the IC_{50} of resveratrol for proliferation inhibition was 34 μM, while it was of 59 μM for normal haematopoietic cells (Gautam et al., 2000). Similarly, SV40-transformed human fibroblasts (WI38VA) were shown to be more sensitive to resveratrol than normal fibroblasts in modulating expression of pro- versus anti-apoptotic genes (Lu et al., 2001; Gosslau et al., 2005). In accordance with the two cases mentioned above, resveratrol had specific cytotoxic effects toward hepatic tumour HepG2 cells, compared to normal human hepatocytes (Lancon et al., 2004).

After oral ingestion, the liver is one of the primary targets of resveratrol and its metabolites, resveratrol-3-glucuronide and resveratrol-3-sulphate, in rodents and in humans (Bertelli et al., 1998; Vitrac et al., 2003; Sale et al., 2004). As revealed by several studies, different sub-populations of hepatocytes are involved in proliferation elicited in response to various types of liver injury in rodents (Styles et al., 1988). In rats and mice, polyploidization affects most of the hepatocytes and is a progressive phenomenon that continues throughout the life of the animal. In rats, after 10 weeks of life, the liver consists mainly of tetraploid cells (2 × 2n and 4n) and some octoploids (2 × 4n and 8n) (Alfert and Geschwind, 1958; Brodsky and Uryvaeva, 1977; Bohm and Noltemeyer 1981; Severin et al., 1984). In primary rat hepatocyte cultures most cells are tetraploid with a small fraction of diploid and octoploid cells. Approximately 30 % of the cells are bi-nucleated with two tetraploid or two diploid nuclei (Eckl, 1993). These cells are a consequence of DNA synthesis and mitosis with failure in cell division (Epstein, 1967; Wheatley, 1972).

In order to establish whether resveratrol affected proliferation in primary rat hepatocyte cultures, we determined the effect of this compound on DNA synthesis

Correspondending author: Juan A. Rubiolo, Departamento de Fisiología, Facultad de Veterinaria, Universidad de Santiago de Compostela, 27002 Lugo, Spain. Phone & Fax: (+34) 982-252231-Ext: 22217; e-mail: ja.rubiolo@usc.es

Abbreviations: cAMP – cyclic adenosine monophosphate, EGF – epidermal growth factor.

and cell cycle of hepatocyte cultures. We also performed studies with an immortalized rat hepatic cell line (Clone 9) in order to determine whether resveratrol affected these processes differentially in normal and transformed cells.

Material and Methods

Resveratrol, 99%, was purchased from Sigma (Madrid, Spain, product code R5010). It was dissolved in DMSO (Panreac, Barcelona, Spain) and stored at -20 °C. When added to cell cultures, the final DMSO concentration was 0.1 % or lower.

Cell culture

Primary rat hepatocyte isolation and culture

All of the experimental protocols conformed to the ethics guidelines of the Santiago de Compostela University. Primary rat hepatocytes were obtained from 200–300 g Sprague-Dawley male rats (Charles River, Barcelona, Spain), fed *ad libitum*. The Seglen perfusion method was followed with modifications (Berry and Friend, 1969, Seglen, 1976). Rats were anaesthetized with a ketamine : xylazine mix (42.5% : 20%, Ketolar® 50 mg, Parke Davis (Madrid, Spain); Rompun® 2%, Bayer (Madrid, Spain)) in physiological solution. After perfusion, the liver was extracted and the cells were dispersed in Leibovitz medium (Sigma). The cells were filtered and allowed to decant for 15 min. The cell pellet was washed twice with the same medium and cell viability was determined by trypan blue exclusion. Preparations with less than 80 % of viability were discarded. The cells were resuspended in attachment medium [199 : E-MEM 1 : 4 (Sigma), 5 mg/l insulin (Sigma), 26.2 mM HCO_3Na (Panreac), 100 µg/ml streptomycin (Sigma), 100 UI/ml penicillin (Calbiochem, part of Merk-Millipore, Madrid, Spain), 1.2 µM dexamethasone (Sigma), 1 g/l BSA (Sigma), 10 % foetal bovine serum (Gibco, part of Invitrogen, Madrid, Spain)]. The cells were plated at a density of 7.2×10^4 cells/cm^2 on 96-well plates (Nunclon™, Sigma) and incubated at 37 °C and 5 % CO_2 during 5 h. After this period the medium was changed to post-attachment medium [199 : E-MEM 1 : 4 (Sigma), 5 mg/l insulin (Sigma), 26.2 mM HCO_3Na (Panreac), 100 µg/ml streptomycin (Sigma), 100 UI/ml penicillin (Calbiochem), 0.6 mM hydroxycortisone (Sigma), 1 g/l BSA (Sigma), 10% foetal bovine serum (Gibco)]. Cells were maintained in this medium at 37 °C in 5% CO_2 atmosphere for different time periods depending on the experiment.

Clone-9 cell culture

The rat liver transformed cell line, Clone-9, was purchased from the European Collection of Cell Cultures, Ref. N° 88072203. Cells were cultured with Ham F12-K medium (Sigma) supplemented with 0.25 % HCO_3Na (Panreac), 10 % foetal bovine serum (Gibco), 100 UI/ml penicillin (Sigma) and 100 mg/ml streptomycin (Sigma) at 37 °C in 5 % CO_2 atmosphere.

Proliferation determination

5-Bromo-2´-deoxy-uridine (dBrU) incorporation

To determine DNA synthesis, the commercial kit 5-Bromo-2´-deoxy-uridine Labeling and Detection Kit III (Roche®, Barcelona, Spain) was used.

Primary rat hepatocytes were seeded in 96-well plates. Twenty-four hours after plating, the medium was changed and 10 ng/ml of epithelial growth factor (Sigma, EGF) was added, except for control cells. Cells were incubated with EGF for another 24 h to induce proliferation. After this time period the medium was changed and 10 ng/ml EGF, 10 µM dBrU and 10, 25, 50 and 75 µM resveratrol or vehicle (DMSO) were added. After 24 h, dBrU incorporation was determined following the kit instructions and plotted as a measure of the absorbance determined for the different treatments.

Clone-9 cells were seeded in 96-well plates. Cells, confluent at 80 %, were treated with 25, 50 and 75 µM resveratrol or vehicle (DMSO) for 24 h. After this time period, dBrU incorporation was measured following the kit instructions.

Total DNA determination

Primary rat hepatocytes were treated with EGF plus resveratrol or vehicle and Clone-9 cells with resveratrol or vehicle in the same way as they were treated for the dBrU incorporation assay. After these treatments the cells were lysed with TNE buffer [150 mM NaCl (Panreac), 25 mM Tris-HCl (Sigma) pH 7.8, 1 mM EDTA (Sigma), and 1 % SDS (Sigma)]. A standard curve was generated spectrofluorimetrically using 0–800 ng of salmon sperm DNA diluted in a solution of PBS with 1/20000 Hoechst 33258 (Sigma, λ_{ex}=356; λ_{em}=458) and 2 M NaCl (Panreac), pH 7.4. Ten µl of the cell lysates were incubated in the Hoechst 33258 solution in the same conditions as the standards during 30 min. Finally, the DNA concentrations in the cell lysates were determined measuring the fluorescence of each sample and extrapolating the DNA concentration from the standard curve.

Flow cytometry

Primary hepatocytes or Clone-9 cells treated for 24 h with vehicle, 25, 50 or 75 µM resveratrol and EGF were detached from the plates with Accutase (PAA, Colbe, Germany) and washed twice with PBS. Cells were fixed with 70% ethanol for 30 min at 4 °C. Fixed cells were washed with PBS and resuspended in a solution of PBS with 100 µg/ml propidium iodide (Sigma) and 0.5 mg/ml RNAse (Sigma). After 30 min of incubation in this solution 5,000 events were captured with an AMNIS ImageStream (Seattle, WA) imaging flow cytometer and the analysis was performed with the IDEAS® 3.0 Cell Image Analysis Software (AMNIS).

Statistical analysis

One-way ANOVA was employed for comparison of significant differences among groups. Comparisons be-

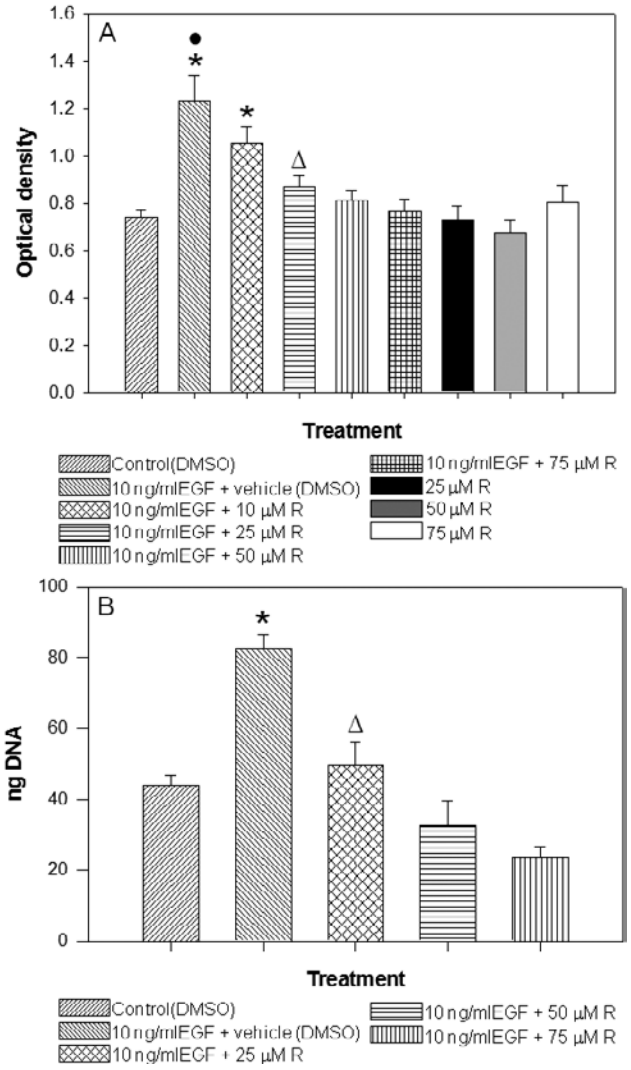

Fig. 1. **(A)** Proliferation determination in primary rat hepatocyte cultures by dBrU incorporation.
* Significant differences between cells treated with EGF + vehicle (DMSO) or R 10 µM and the rest of the treatments (N = 4, P < 0.01). Δ Significant difference with respect to control (N = 4, P < 0.05).
● Significant difference with respect to the cells treated with EGF + 10 µM R (N = 4, P < 0.01).
(B) Proliferation determination in primary rat hepatocyte cultures by total DNA determination.
* Significant difference with respect to the rest of the treatments (N = 4, P < 0.01). Δ Significant difference with respect to the cells treated with EGF + 50 or 75 µM R (N = 4, P < 0.01).

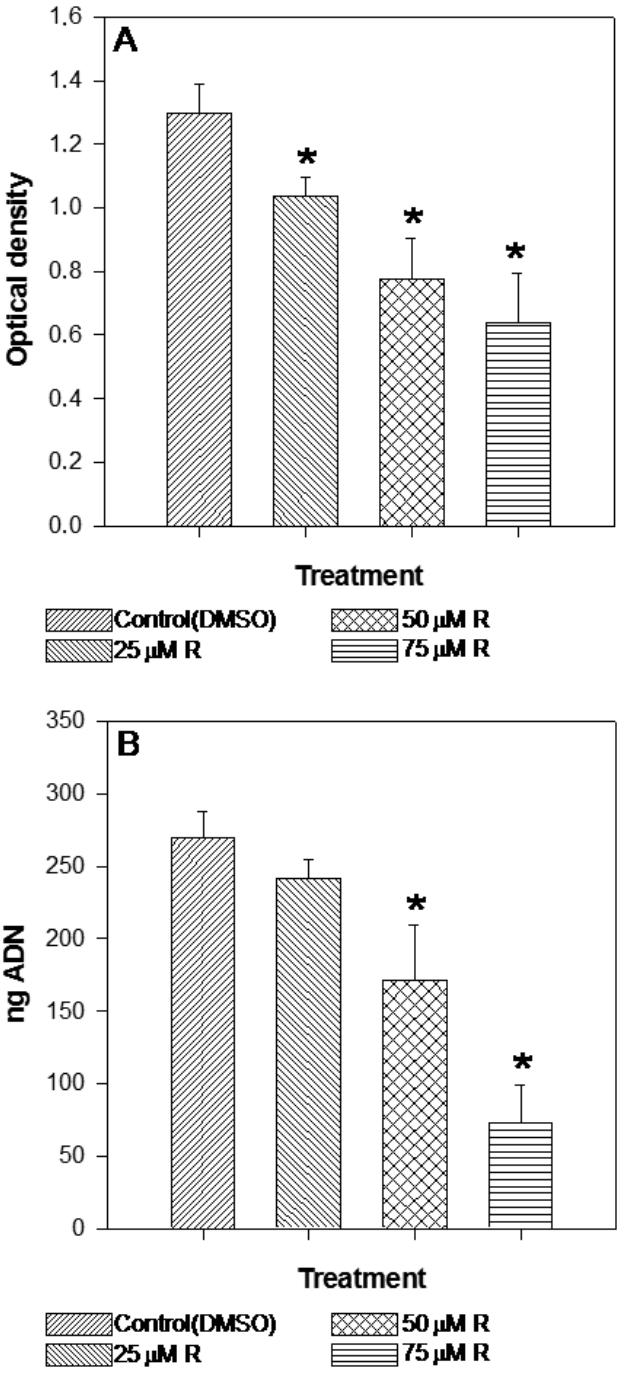

Fig. 2. **(A)** Proliferation determination in Clone-9 cell cultures by dBrU incorporation. **(B)** Proliferation determination in Clone-9 cell cultures by total DNA determination.
* Significant differences with respect to control cells (N = 4, P < 0.01).

tween groups were made by the Holm-Sidak multiple-range test. Values are presented as the mean ± SD. The value of P < 0.05 (N = 4) was considered significant.

Results

Primary rat hepatocytes treated with EGF + 10, 25, 50 and 75 µM resveratrol showed lower incorporation of dBrU (Fig. 1A) and less total DNA (Fig. 1B) than cells treated with EGF + vehicle. No significant changes in dBrU incorporation were observed in the cells treated with EGF + 25, 50 and 75 µM resveratrol with respect to control cells without EGF (Fig. 1A). When primary rat hepatocytes were treated with resveratrol and without EGF, no increase in dBrU incorporation was observed when compared to control cells treated with the vehicle (Fig. 1A).

When Clone-9 cells were treated with 25, 50 and 75 µM resveratrol, lower incorporation of dBrU was ob-

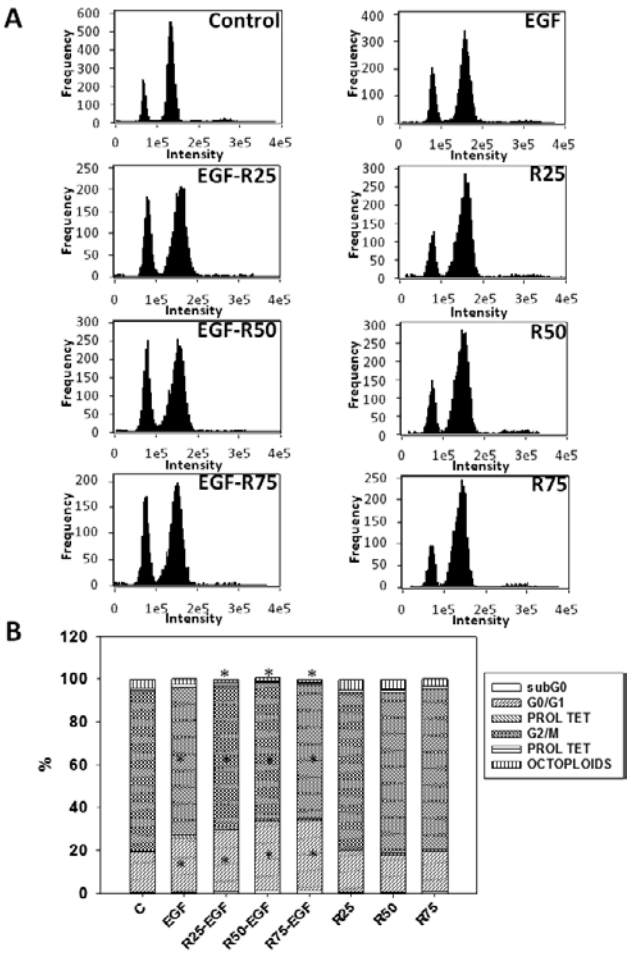

Fig. 3. **(A)** Typical histograms obtained in vehicle-, resveratrol-, EGF- or resveratrol+EGF-treated cultures of primary hepatocytes as described in Material and Methods. **(B)** Cell cycle analysis after flow cytometry as described in A. * Significant differences with respect to control or resveratrol-treated cells (N = 3, P < 0.01).

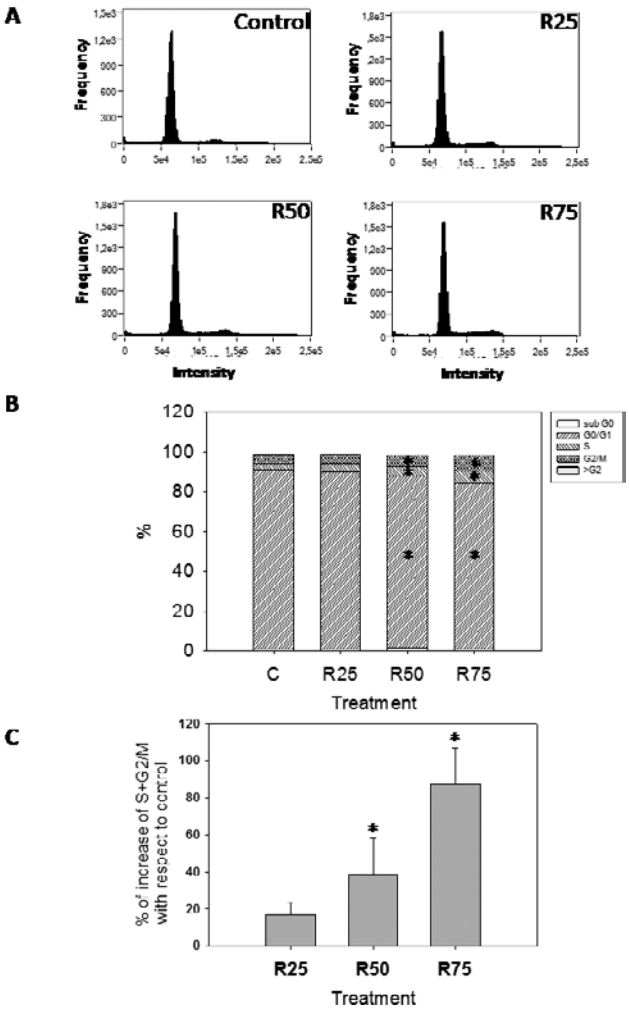

Fig. 4. **(A)** Typical histograms obtained in vehicle- or resveratrol-treated cells as described in Material and Methods. **(B)** Cell cycle analysis after flow cytometry as described in A. **C)** Summary of the G1 and S phases showing a dose-dependent increase of the cells in these cell cycle phases in resveratrol-treated cells.
* Significant differences with respect to control cells (N = 3, P < 0.01).

served in a dose-dependent manner compared to cells treated with the vehicle (Fig. 2A). The same was observed when total DNA was determined (Fig. 2B).

When the cell cycle of primary rat hepatocytes was analysed in resveratrol-, EGF+resveratrol, or vehicle-treated cells (typical histograms obtained are presented in Fig. 3A), an arrest in G0/G1, in a dose-dependent manner, was observed. This was accompanied with a decrease in the G2/M population. Also, a decrease in the octoploid cell population was observed in EGF+resveratrol-treated cells (Fig. 3B). No effect was observed when hepatocytes were treated with resveratrol alone in the same concentrations used when hepatocytes were induced to proliferate with EGF (Fig. 3B). On the contrary, when Clone-9 cells were treated with resveratrol (typical histograms obtained are shown in Fig. 4A), an arrest of the cell cycle in G2/M was observed (Fig. 4B). Also, an increase in the cells in the S phase was observed after resveratrol treatment, which is explained by DNA synthesis inhibition (Fig. 4B). This inhibition together with that in G2/M represented a dose-dependent increase of approximately 20, 40 and 80 % in 25, 50 and 75 μM treated cells when compared to controls (Fig. 4C).

Discussion

In this work we show that resveratrol inhibits proliferation in primary rat hepatocytes as well as in transformed rat hepatic cells in a dose-dependent manner. We didn't observe the higher sensitivity towards inhibition of cell proliferation in transformed cells compared to normal cells reported by others (Gautam et al., 2000; Lu et al., 2001; Gosslau et al., 2005). Resveratrol had no effect on the proliferation of quiescent primary hepatocytes.

Cell cycle analysis showed that resveratrol inhibits primary rat hepatocyte proliferation in G0/G1. This indicates that after mitogenic stimulation, the cells in G2,

which are the most abundant in normal rat liver, can proceed through mitosis, but after reaching G1, they are incapable of progressing to S because of DNA synthesis inhibition. Resveratrol has been shown to induce an increase in the levels of cAMP in MCF-7 human breast cancer cells (El-Mowafy and Alkhalaf, 2003), and recently the direct interaction of this molecule with cAMP-degrading phosphodiesterases, leading to elevated cAMP levels, has been reported (Park et al., 2012). Considering that cyclic adenosine monophosphate (cAMP) and agents that increase intra-cellular cAMP inhibit hepatocyte proliferation induced by EGF (Bronstad et al., 1983; Vintermyr et al., 1989; Refsnes et al., 1992), resveratrol could inhibit primary hepatocyte proliferation by this mechanism. Supporting this is the fact that cAMP inhibits the G0/G1 to S transition in primary hepatocytes (Vintermyr et al., 1989), which is what we observed for this type of cells.

In transformed rat hepatic cells, a mild cell cycle inhibition was observed, in this case in G2/M, indicating that besides the inhibition of DNA synthesis observed in the presence of resveratrol, which appears as an accumulation of cells in S phase, this compound has also an effect on the tetraploid cells, at the level of progression from G2 to M, and/or on the chromosome segregation and cell division. In transformed cells, resveratrol has been shown to induce cell cycle arrest, affecting the S to G2 transition (Ragione et al., 1998), through a p53-dependent pathway (Huang et al., 1999; Liontas and Yeger 2004). This protein could be involved in the cell cycle arrest of Clone-9 cell reported in this work, given that these cells possess wild-type p53. Also, the flow cytometric analysis showed an increase of the S and G2 populations in resveratrol-treated Clone-9 cells, pointing to S to G2 cell cycle transition inhibition.

The low micromolar concentrations of resveratrol employed in the present studies are similar to those used by others in the investigation of the biological effects of resveratrol in cultured cells (Fremont, 2000; Gusman et al., 2001; Wallerath et al., 2002; Dong 2003). Whether these concentrations can be reached *in vivo* in the liver cannot be answered at present. There are studies indicating that because of its high lipophilicity, resveratrol can accumulate in tissues, such as heart, liver, and kidney after oral administration in wine (Gusman et al., 2001). Studies in animal models (mice, rats and dogs) and humans have shown that resveratrol undergoes rapid metabolism in the gut to glucuronide and sulphate conjugates, which are well absorbed and result in high circulating levels (Yu et al., 2002; Walle et al., 2006). Glucuronidation and sulphation of resveratrol may be important determinants of its pharmacological activity because resveratrol glucuronide or sulphate conjugates may be deconjugated at the target sites of action, releasing the substrate to elicit biological activity (Kuhnle et al., 2000; Walle et al., 2004; Signorelli and Ghidoni, 2005). Furthermore, the biologically active resveratrol sulphate or glucuronide cannot be ruled out. It has been observed that after an oral dose of red wine containing 26 μg of resveratrol, a 10^{-4} molar concentration of this compound can be reached in the liver and the kidneys of rats for more than 2 h (Bertelli et al., 1996). *In vivo* studies have demonstrated that resveratrol can prevent the decrease in the activities of catalase, superoxide dismutase, glutathione peroxidase and glutathione reductase in the livers of rats treated with ethanol (Kasdallah-Grissa et al., 2007). *In vitro* resveratrol exerts a cytoprotective action and induces an increase in the enzymatic antioxidant response in primary rat hepatocytes (Rubiolo and Vega, 2008; Rubiolo et al., 2008). These observations point to a beneficial effect of this molecule on human health. In normal livers there is a very low level of hepatocyte proliferation, but after liver damage hepatocytes proliferate until the liver mass is restored (Fausto and Campbell, 2003). Although the results obtained in this work cannot be directly related to an *in vivo* situation, the possibility exists that resveratrol could be harmful for subjects with liver damage. It should be noted that resveratrol, besides being present in some foods and beverages, is available as a diet supplement, and not as a prescribed drug, in several formats (pills, creams, etc.) for human consumption as an antioxidant and age-prolonging compound.

References

Ahmad, N., Adhami, V. M., Afaq, F., Feyes, D. K., Mukhtar, H. (2001) Resveratrol causes WAF-1/p21-mediated G(1)-phase arrest of cell cycle and induction of apoptosis in human epidermoid carcinoma A431 cells. *Clin. Cancer Res*. **7**, 1466-1473.

Alfert, M., Geschwind, I. L. (1958) The development of polysomaty in rat liver. *Exp. Cell Res*. **15**, 230-232.

Berry, M., Friend, D. (1969) High-yield preparation of isolated rat liver parenchymal cells. *J. Cell. Biol*. **43**, 506-520.

Bertelli, A., Bertelli, A. A., Gozzini, A., Giovannini, L. (1998) Plasma and tissue resveratrol concentrations and pharmacological activity. *Drugs Exp. Clin. Res*. **24**, 133-138.

Bertelli, A. A., Giovannini, L., Stradi, R., Bertelli, A., Tillement, J. P. (1996) Plasma, urine and tissue levels of trans- and cis-resveratrol (3,4',5-trihydroxystilbene) after short-term or prolonged administration of red wine to rats. *Int. J. Tissue React*. **18**, 67-71.

Bohm, N., Noltemeyer, N. (1981) Development of binuclearity and DNA-polyploidization in the growing mouse liver. *Histochemistry*. **72,** 55-61.

Brodsky, W. Y., Uryvaeva, I. V. (1977) Cell polyploidy: its relation to tissue growth and function. *Int. Rev. Cytol*. **50**, 275-332.

Bronstad, G. O., Sand, T. E., Christoffersen, T. (1983) Bidirectional concentration-dependent effects of glucagon and dibutyryl cyclic AMP on DNA synthesis in cultured adult rat hepatocytes. *Biochim. Biophys. Acta* **763**, 58-63.

Delmas, D., Passilly-Degrace, P., Jannin, B., Cherkaoui Malki, M., Latruffe, N. (2002) Resveratrol, a chemopreventive agent, disrupts the cell cycle control of human SW480 colorectal tumor cells. *Int. J. Mol. Med*. **10**,193-199.

Dong, Z. (2003) Molecular mechanism of the chemopreventive effect of resveratrol. *Mutat. Res*. **523-524**, 145-150.

Eckl, P. M. (1993) Occurrence and possible consequences of multipolar mitoses in primary cultures of adult rat hepatocytes. *J. Cell. Physiol*. **154**, 601-607.

El-Mowafy, A. M., Alkhalaf, M. (2003) Resveratrol activates adenylyl-cyclase in human breast cancer cells: a novel, estrogen receptor-independent cytostatic mechanism. *Carcinogenesis* **24**, 869-873.

Epstein, C. J. (1967) Cell size, nuclear content, and the development of polyploidy in the mammalian liver. *Proc. Natl. Acad. Sci. USA* **57**, 327-334.

Estrov, Z., Shishodia, S., Faderl, S., Harris, D., Van, Q., Kantarjian, H. M., Talpaz, M., Aggarwal, B. B. (2003) Resveratrol blocks interleukin-1β-induced activation of the nuclear transcription factor NF-κB, inhibits proliferation, causes S-phase arrest, and induces apoptosis of acute myeloid leukemia cells. *Blood* **102**, 987-995.

Fausto, N., Campbell, J. S. (2003) The role of hepatocytes and oval cells in liver regeneration and repopulation. *Mech. Dev*. **120**, 117-130.

Fremont, L. (2000) Biological effects of resveratrol. *Life Sci*. **66**, 663-673.

Gautam, S. C., Xu, Y. X., Dumaguin, M., Janakiraman, N., Chapman, R. A. (2000) Resveratrol selectively inhibits leukemia cells: a prospective agent for ex vivo bone marrow purging. *Bone Marrow Transplant*. **25**, 639-645.

Gosslau, A., Chen, M., Ho, C. T., Chen, K. Y. (2005) A methoxy derivative of resveratrol analogue selectively induced activation of the mitochondrial apoptotic pathway in transformed fibroblasts. *Br. J. Cancer* **92**, 513-521.

Gusman, J., Malonne, H., Atassi, G. (2001) A reappraisal of the potential chemopreventive and chemotherapeutic properties of resveratrol. *Carcinogenesis* **22**, 1111-1117.

Hsieh, T. C., Wu, J. M. (1999) Differential effects on growth, cell cycle arrest, and induction of apoptosis by resveratrol in human prostate cancer cell lines. *Exp. Cell. Res*. **249**, 109-115.

Huang, C., Ma, W. Y., Goranson, A., Dong, Z. (1999) Resveratrol suppresses cell transformation and induces apoptosis through a p53-dependent pathway. *Carcinogenesis* **20**, 237-242.

Ignatowicz, E., Baer-Dubowska, W. (2001) Resveratrol, a natural chemopreventive agent against degenerative diseases. *Pol. J. Pharmacol*. **53**, 557-569.

Kasdallah-Grissa, A., Mornagui, B., Aouani, E., Hammami, M., El May, M., Gharbi, N., Kamoun, A., El-Fazaa, S. (2007) Resveratrol, a red wine polyphenol, attenuates ethanol-induced oxidative stress in rat liver. *Life Sci.* **80**, 1033-1039.

Kuhnle, G., Spencer, J. P., Chowrimootoo, G., Schroeter, H., Debnam, E. S., Srai, S. K., Rice-Evans, C., Hahn, U. (2000) Resveratrol is absorbed in the small intestine as resveratrol glucuronide. *Biochem. Biophys. Res. Commun*. **272**, 212-217.

Lancon, A., Delmas, D., Osman, H., Thenot, J. P., Jannin, B., Latruffe, N. (2004) Human hepatic cell uptake of resveratrol: involvement of both passive diffusion and carrier-mediated process. *Biochem. Biophys. Res. Commun*. **316**, 1132-1137.

Liang, Y. C., Tsai, S. H., Chen, L., Lin-Shiau, S. Y., Lin, J. K. (2003) Resveratrol-induced G2 arrest through the inhibition of CDK7 and p34CDC2 kinases in colon carcinoma HT29 cells. *Biochem. Pharmacol*. **65**, 1053-1060.

Liontas, A., Yeger, H. (2004) Curcumin and resveratrol induce apoptosis and nuclear translocation and activation of p53 in human neuroblastoma. *Anticancer Res*. **24**, 987-998.

Lu, J., Ho, C. H., Ghai, G., Chen, K. Y. (2001) Resveratrol analog, 3,4,5,4'-tetrahydroxystilbene, differentially induces pro-apoptotic p53/Bax gene expression and inhibits the growth of transformed cells but not their normal counterparts. *Carcinogenesis* **22**, 321-328.

Mgbonyebi, O. P., Russo, J., Russo, I. H. (1998) Antiproliferative effect of synthetic resveratrol on human breast epithelial cells. *Int. J. Oncol*. **12**, 865-869.

Park, S. J., Ahmad, F., Philp, A., Baar, K., Williams, T., Luo, H., Ke, H., Rehmann, H., Taussig, R., Brown, A. L., Kim, M. K., Beaven, M. A., Burgin, A. B., Manganiello, V., Chung, J. H. (2012) Resveratrol ameliorates aging-related metabolic phenotypes by inhibiting cAMP phosphodiesterases. *Cell* **148**, 421-433.

Ragione, F. D., Cucciolla, V., Borriello, A., Pietra, V. D., Racioppi, L., Soldati, G., Manna, C., Galletti, P., Zappia, V. (1998) Resveratrol arrests the cell division cycle at S/G2 phase transition. *Biochem. Biophys. Res. Commun*. **250**, 53-58.

Refsnes, M., Thoresen, G. H., Sandnes, D., Dajani, O. F., Dajani, L., Christoffersen, T. (1992) Stimulatory and inhibitory effects of catecholamines on DNA synthesis in primary rat hepatocyte cultures: role of α1- and β-adrenergic mechanisms. *J. Cell. Physiol*. **151**, 164-171.

Rubiolo, J. A., Mithieux, G., Vega, F. V. (2008) Resveratrol protects primary rat hepatocytes against oxidative stress damage: activation of the Nrf2 transcription factor and augmented activities of antioxidant enzymes. *Eur. J. Pharmacol*. **591**, 66-72.

Rubiolo, J. A., Vega, F. V. (2008) Resveratrol protects primary rat hepatocytes against necrosis induced by reactive oxygen species. *Biomed. Pharmacother*. **62**, 606-612.

Sale, S., Verschoyle, R. D., Boocock, D., Jones, D. J., Wilsher, N., Ruparelia, K. C., Potter, G. A., Farmer, P. B., Steward, W. P., Gescher, A. J. (2004) Pharmacokinetics in mice and growth-inhibitory properties of the putative cancer chemopreventive agent resveratrol and the synthetic analogue trans 3,4,5,4'-tetramethoxystilbene. *Br. J. Cancer* **90**, 736-744.

Seglen, P. O. (1976) Preparation of isolated rat liver cells. *Methods Cell. Biol*. **13**, 29-83.

Severin, E., Willers, R., Bettecken, T. (1984) Flow cytometric analysis of mouse hepatocyte ploidy. II. The development of polyploidy pattern in four mice strains with different life spans. *Cell Tissue Res*. **238**, 649-652.

Signorelli, P., Ghidoni, R. (2005) Resveratrol as an anticancer nutrient: molecular basis, open questions and promises. *J. Nutr. Biochem*. **16**, 449-466.

Styles, J. A., Kelly, M., Pritchard, N. R., Elcombe, C. R. (1988) A species comparison of acute hyperplasia induced by the peroxisome proliferator methylclofenapate: involvement of the binucleated hepatocyte. *Carcinogenesis* **9**, 1647-1655.

Vintermyr, O. K., Mellgren, G., Boe, R., Doskeland, S. O. (1989) Cyclic adenosine monophosphate acts synergistically with dexamethasone to inhibit the entrance of cultured adult rat hepatocytes into S-phase: with a note on the use of nucleolar and extranucleolar [3H]-thymidine labelling patterns to determine rapid changes in the rate of onset of DNA replication. *J. Cell. Physiol*. **141**, 371-382.

Vitrac, X., Desmouliere, A., Brouillaud, B., Krisa, S., Deffieux, G., Barthe, N., Rosenbaum, J., Merillon, J. M. (2003) Distribution of [14C]-trans-resveratrol, a cancer chemopreventive polyphenol, in mouse tissues after oral administration. *Life Sci*. **72**, 2219-2233.

Walle, T., Hsieh, F., DeLegge, M. H., Oatis, J. E., Jr., Walle, U. K. (2004) High absorption but very low bioavailability of oral resveratrol in humans. *Drug Metab. Dispos*. **32**, 1377-1382.

Walle, T., Walle, U. K., Sedmera, D., Klausner, M. (2006) Benzo[A]pyrene-induced oral carcinogenesis and chemoprevention: studies in bioengineered human tissue. *Drug Metab. Dispos*. **34**, 346-350.

Wallerath, T., Deckert, G., Ternes, T., Anderson, H., Li, H., Witte, K., Forstermann, U. (2002) Resveratrol, a polyphenolic phytoalexin present in red wine, enhances expression and activity of endothelial nitric oxide synthase. *Circulation* **106**, 1652-1658.

Wheatley, D. N. (1972) Binucleation in mammalian liver. Studies on the control of cytokinesis in vivo. *Exp. Cell. Res*. **74**, 455-465.

Yu, C., Shin, Y. G., Chow, A., Li, Y., Kosmeder, J. W., Lee, Y. S., Hirschelman, W. H., Pezzuto, J. M., Mehta, R. G., van Breemen, R. B. (2002) Human, rat, and mouse metabolism of resveratrol. *Pharm. Res*. **19**, 1907-1914.