

Oestrogen inhibits resveratrol-induced post-translational modification of p53 and apoptosis in breast cancer cells

S Zhang^{1,4}, HJ Cao^{1,4}, FB Davis^{*1}, H-Y Tang², PJ Davis^{1,2,3} and H-Y Lin²

¹The Ordway Research Institute, 150 New Scotland Avenue, Albany, NY 12208, USA; ²The Research Service, Stratton Veterans Administration Medical Center, 113 Holland Avenue, Albany, NY 12208, USA; ³The Wadsworth Center, New York State Department of Health, Empire State Plaza, Albany, NY 12201, USA

Resveratrol, a naturally occurring stilbene, induced apoptosis in human breast cancer MCF-7 cells. The mechanism of this effect was dependent on mitogen-activated protein kinase (MAPK, ERK1/2) activation and was associated with serine phosphorylation and acetylation of p53. Treatment of MCF-7 cells with resveratrol in the presence of 17 β -oestradiol (E₂) further enhanced MAPK activation, but E₂ blocked resveratrol-induced apoptosis, as measured by nucleosome ELISA and DNA fragmentation assays. E₂ inhibited resveratrol-stimulated phosphorylation of serines 15, 20 and 392 of p53 and acetylation of p53 in a concentration- and time-dependent manner. These effects of E₂ on resveratrol action were blocked by ICI 182,780 (ICI), an inhibitor of the nuclear oestrogen receptor- α (ER). ICI 182,780 did not block the actions of resveratrol, alone. Electrophoretic mobility studies of p53 binding to DNA and of *p21* expression indicated that E₂ inhibited resveratrol-induced, p53-directed transcriptional activity. These results suggest that E₂ inhibits p53-dependent apoptosis in MCF-7 cells by interfering with post-translational modifications of p53 which are essential for p53-dependent DNA binding and consequent stimulation of apoptotic pathways. These studies provide insight into the complex pathways by which apoptosis is induced by resveratrol in E₂-depleted and -repleted environments.

British Journal of Cancer (2004) 91, 178–185. doi:10.1038/sj.bjc.6601902 www.bjcancer.com

Published online 8 June 2004

© 2004 Cancer Research UK

Keywords: breast cancer; MCF-7 cells; p53; resveratrol; oestrogen; apoptosis; mitogen-activated protein kinase

Resveratrol is a phytoalexin that occurs naturally in grape skin and several medicinal plants (Jeanet *et al*, 1991; Lu and Serrero, 1999). It has anticancer (Jang *et al*, 1997) and other biologic properties (Pace-Asciak *et al*, 1995). The mechanism of the antitumour effect of resveratrol is not well understood, but in some tumour cell models induction of apoptosis is involved (Clement *et al*, 1998; Huang *et al*, 1999; She *et al*, 2001). We have demonstrated resveratrol-induced apoptosis in four thyroid cancer cell lines (Shih *et al*, 2002) and two prostate cancer cell lines (Lin *et al*, 2002). Resveratrol has been shown to exert mixed oestrogen agonist/antagonist activities in mammary cancer cell cultures, but in the presence of 17 β -oestradiol (oestradiol, E₂), resveratrol may function as an antioestrogen (Bhat *et al*, 2001), antagonising E₂ activities. In this study, we have explored the effects of resveratrol and E₂, both separately and together, in breast cancer (MCF-7) cells, and show that E₂ inhibits resveratrol-induced apoptosis in these cells.

p53 is an oncogene suppressor protein that is present at low levels in normal cells (Higashimoto *et al*, 2000). In response to a variety of stresses, including DNA damage (Higashimoto *et al*, 2000), levels of cellular p53 protein rise; this increase in p53 results at least in part from a post-translational mechanism that stabilises the protein (Ashcroft and Vousden, 1999). p53 can adopt two

forms *in vitro*, a latent form that binds naked DNA poorly and an active form that binds to DNA well. Conversion of the latent form to the active form is thought to occur by an allosteric mechanism conditioned by phosphorylation and acetylation (Sakaguchi *et al*, 1998; Liu *et al*, 1999; Prives and Manley, 2001). Serine phosphorylation of wild-type p53 at different sites has different biological consequences (Agarwal *et al*, 1998). We and others have shown that resveratrol-induced serine phosphorylation of p53 in various cancer cells is essential to resveratrol-induced apoptosis (She *et al*, 2001; Lin *et al*, 2002; Shih *et al*, 2002). Acetylation of p53 occurs in the wake of serine phosphorylation and is essential for p53-dependent gene expression (Berlev *et al*, 2001). In this report, we show that although both E₂ and resveratrol stimulate mitogen-activated protein kinase (MAPK; ERK1/2) activity in an additive manner in MCF-7 breast cancer cells, E₂ *inhibits* resveratrol-induced serine phosphorylation and acetylation of p53, as well as oligonucleotide binding of p53. As a result, resveratrol-induced p53-dependent *p21* gene expression and apoptosis are inhibited by E₂ in these breast cancer cells.

MATERIALS AND METHODS

Cell lines and reagents

The MCF-7 human breast cancer cells were provided by Dr J Bennett (Albany Medical College, Albany, NY, USA). Cell lines were maintained for study in DMEM supplemented with 10% foetal bovine serum (FBS), in a 5% CO₂/95% O₂ incubator at 37°C. Cells were placed in 0.25% serum-supplemented medium for 2

*Correspondence: Dr FB Davis; E-mail: fdavis@ordwayresearch.org

⁴These authors contributed equally to this work

Received 23 January 2004; revised 14 April 2004; accepted 19 April 2004; published online 8 June 2004

days prior to treatment. The serum used for these studies was previously depleted of oestradiol by ion exchange resin, resulting in a total E_2 concentration in undiluted serum of $<10^{-11}$ M (Dr Z Cao, New York State Department of Health, Albany, NY, USA, personal communication). Oestradiol and resveratrol were obtained from the Sigma Chemical Company (St Louis, MO, USA), and ICI 182,780 was obtained from Tocris Cookson Inc. (Ellisville, MO, USA).

Cell fractionation

Fractionation and preparation of nucleoproteins was carried out according to our previously reported methods (Shih *et al*, 2001; Lin *et al*, 2002). Nuclear extracts were prepared by resuspension of the crude nuclei in hypotonic buffer with 420 mM NaCl, 20% glycerol at 4°C with rocking for 1 h, and the supernatants were collected after subsequent centrifugation at 4°C and 13 000 rpm for 10 min.

Immunoblotting

The techniques are standard and have been previously described (Shih *et al*, 2001; Lin *et al*, 2002). Nucleoproteins were separated on discontinuous SDS-PAGE (9% gels), and then transferred by electroblotting to Immobilon membranes (Millipore, Bedford, MA, USA). After blocking with 5% milk in Tris-buffered saline containing 0.1% Tween, the membranes were incubated overnight with polyclonal antibodies to phosphorylated ERK1/2, or serine 6-(ser6-), ser15-, ser20- or ser392-phosphorylated p53 (Cell Signaling, Beverly, MA, USA). A polyclonal antibody to acetylated p53 was from Upstate Biotechnology (Lake Placid, NY, USA). The secondary antibody was goat anti-rabbit IgG (1:1000, Dako, Carpinteria, CA, USA). Immunoreactive proteins were detected by chemiluminescence (Amersham Life Science, Arlington Heights, IL, USA). All immunoblots were scanned and optical densities of bands were measured. Immunoblots shown in the figures are representative of three experiments. Statistical significance of changes in phosphorylation of ERKs 1 and 2, and of p53, as well as p53 acetylation, was calculated by one-way analysis of variance on data from three experiments. Molecular weight markers are included in Figure 1 for reference; band molecular weights are similar to those in Figure 1 in Figures 2–5.

Electrophoretic mobility gel shift assay (EMSA)

Nuclear extracts (10 μ g protein) were incubated in a 25 μ l total reaction volume that contained 10 mM TRIS, pH 7.5, 50 mM NaCl, 1.0 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 4% glycerol and 0.05 μ g μ l⁻¹ poly(dI-dC) (Promega, Madison, WI, USA). A p53-binding oligonucleotide (Santa Cruz, Santa Cruz, CA, USA), labelled with [³²P] by T4 polynucleotide kinase (Promega), was added to the total reaction mixture, which was then incubated for 20 min at room temperature. A control nonreactive SP-1 oligonucleotide (Promega) was prepared in the same manner. Samples were loaded on 4% polyacrylamide gels in low ionic strength buffer (22.3 mM Tris, 22.2 mM borate, 0.5 mM EDTA) and run with cooling at 15 V cm^{-1} . The gels were then dried, exposed to X-ray film and autoradiographs were analysed.

RT-polymerase chain reaction (PCR)

Total RNA was isolated as described previously (Shih *et al*, 2002). First-strand complementary DNAs were synthesised from 1 mg of total RNA using oligo-deoxythymidine and AMV Reverse Transcriptase (Promega). First-strand cDNA templates were amplified for p21 and GAPDH by PCR using a hot start (Ampliwax, Perkin Elmer, Foster City, CA, USA). Primer sequences were as previously

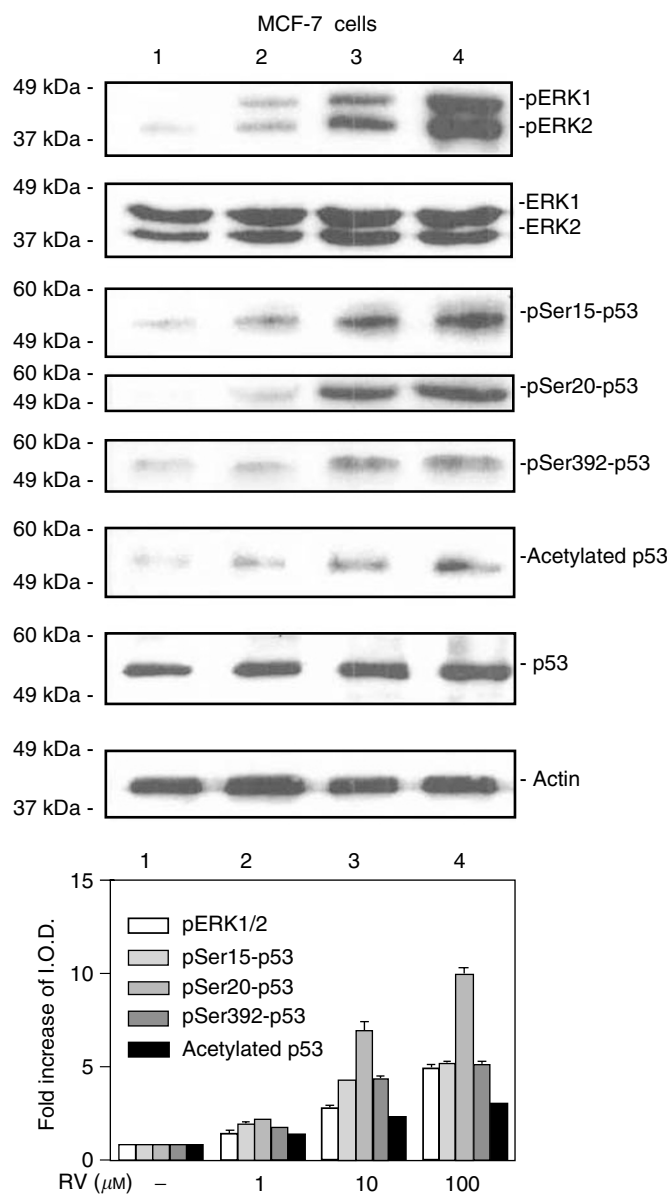


Figure 1 Resveratrol treatment of MCF-7 cells leads to phosphorylation (activation) of MAPK, serine phosphorylation of p53 and acetylation of p53 in MCF-7 breast cancer cells. Cells were treated with 1–100 μ M resveratrol (RV) for 4 h. Nuclear proteins were separated by electrophoresis, and immunoblots performed with selected antibodies. Mitogen-activated protein kinase activation, shown as appearance in nuclei of nuclear phosphorylated ERK1 and ERK2 (pERK1 and 2), and phosphorylation of serines 15, 20 and 392 of p53, as well as acetylation of p53, were induced by resveratrol in a concentration-dependent manner (lanes 2–4). Increases in phosphorylation of ERK1/2 and acetylation and phosphorylation of p53 with 1–100 μ M RV were each significant at $P < 0.001$ by one-way analysis of variance in three similar experiments. The graph below shows the changes in phosphorylation of ERK1/2 and p53, and acetylation of p53, normalized to a value of 1 in control samples, in three experiments. Means \pm s.e.m. are shown. Immunoblots of total nuclear ERKs 1 and 2 (second panel from top) are consistent with activation (phosphorylation) and nuclear accumulation of these proteins, and a slight increase in total nuclear p53 is consistent with the resveratrol effect which we have previously described (Lin *et al*, 2002; Shih *et al*, 2002). Actin immunoblots serve as loading controls in this and subsequent figures. Immunoblots in all figures are representative of three or more experiments. Molecular weights of the proteins shown are as follows: pERK1, 44 kDa; pERK2, 42 kDa; p53, 53 kDa; actin, 43 kDa. (I.O.D., integrated optical density).

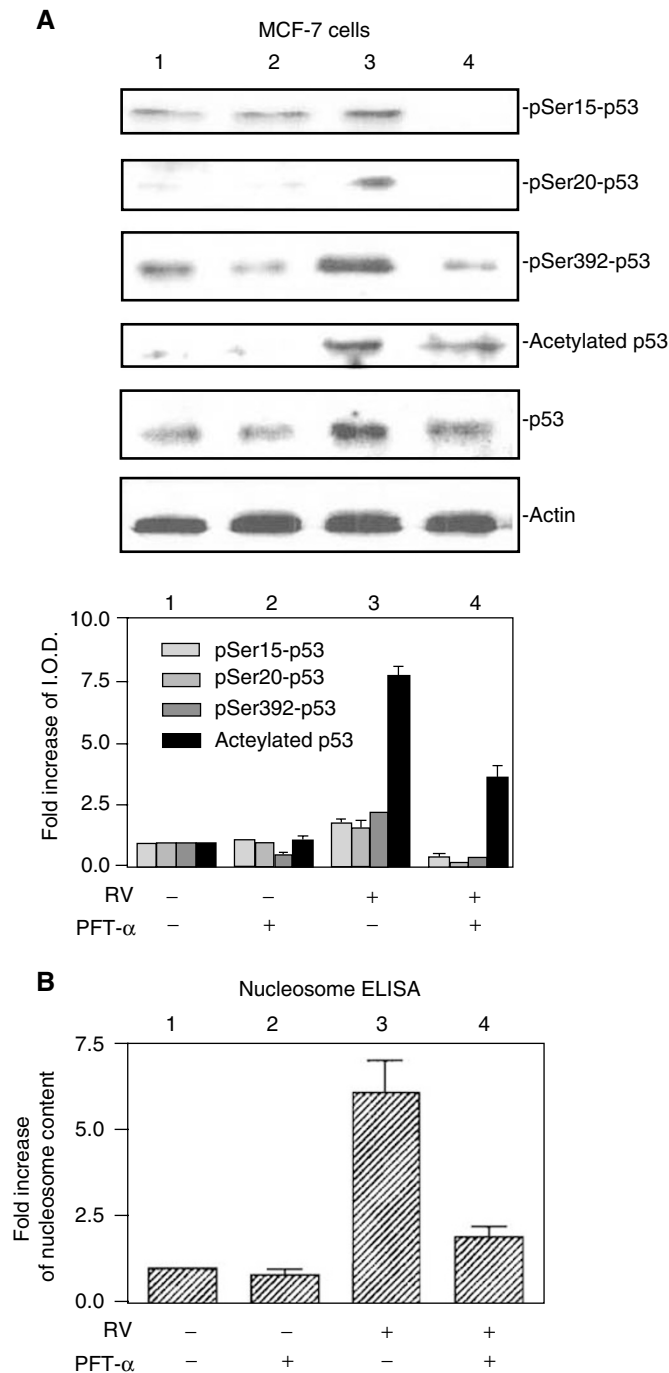


Figure 2 Activation of p53 and apoptosis induced by resveratrol is inhibited by pifithrin- α in MCF-7 cells. **(A)** Cells were treated with $10 \mu\text{M}$ resveratrol (RV) for 4 h in the presence or absence of $20 \mu\text{M}$ pifithrin- α (PFT- α), a specific p53 inhibitor. As shown by immunoblots of nuclear fractions from a representative experiment above, and the accompanying graph below, resveratrol caused nuclear accumulation, serine phosphorylation and acetylation of p53 ($P < 0.05$ comparing lanes 1 and 3 for each parameter). Treatment with PFT- α resulted in decreased phosphorylation and acetylation of p53 ($P < 0.05$ for all parameters, comparing lanes 3 and 4). Molecular weight markers in this figure are not shown, but are similar to those shown in Figure 1. **(B)** Cells were treated with $10 \mu\text{M}$ resveratrol for 24 h in the presence or absence of $20 \mu\text{M}$ PFT- α . Apoptosis, shown by an increase in nucleosome content determined by ELISA, occurred with resveratrol treatment ($P < 0.001$, comparing lanes 1 and 3). Pifithrin- α had no effect alone, but significantly blocked resveratrol-induced apoptosis (comparing lanes 3 and 4, $P < 0.005$). Means \pm s.e.m. of six experiments are shown.

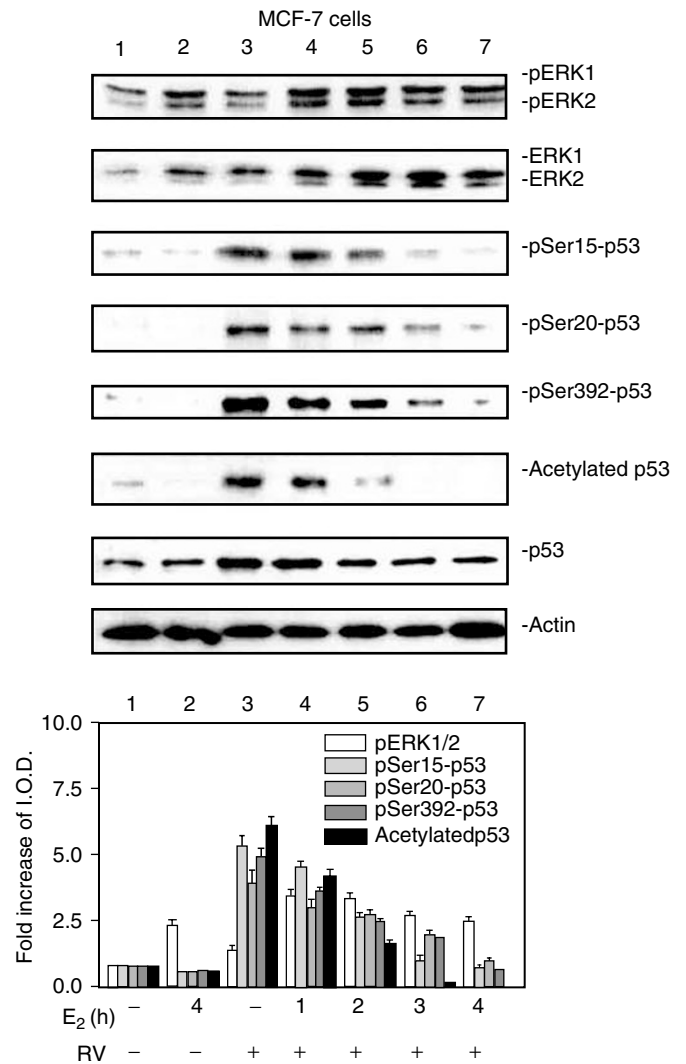


Figure 3 E₂ inhibition of resveratrol-induced activation of MAPK and p53 is time dependent. MCF-7 cells were treated with E₂ (10^{-9} M) alone for 4 h or for different time periods (0–4 h) with resveratrol (RV, $10 \mu\text{M}$, 4 h), after which nuclear fractions were prepared from each sample. This series of immunoblots, representative of three experiments, indicates that minimal activation of ERK1/2 by resveratrol (top panel, lane 3) was enhanced by 1–2 h incubation with E₂ (lanes 4 and 5). A longer incubation with E₂ caused a time-dependent reduction in ERK1/2 phosphorylation (lanes 4–7, $P < 0.05$). Resveratrol induced phosphorylation of serines 15, 20 and 392 of p53 (pSer15-, pSer20- and pSer392-p53), and acetylation of p53 (lane 3 in each panel). However, these effects of resveratrol on p53 post-translational modification were progressively inhibited by coincubation with E₂ for 1–4 h. The reductions in resveratrol-induced serine-15, serine-20, and serine-392 phosphorylation and acetylation of p53 by E₂ were significant at $P < 0.003$ or less. Molecular weight markers in this figure are not shown, but are similar to those shown in Figure 1.

described (Shih *et al*, 2002). The PCR cycle was an initial step of 95°C for 3 min followed by 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, then 25 cycles and a final cycle of 72°C for 8 min. Polymerase chain reaction products were subjected to electrophoresis in 2% agarose gels containing $0.2 \mu\text{g ml}^{-1}$ ethidium bromide. Gels were visualised under UV light and photographed with Polaroid film (Polaroid Co., Cambridge, MA, USA). Photographs were scanned under direct light (Bio-Image, Millipore) for quantitation and illustration. Results of p21 cDNA levels were normalised to GADPH cDNA levels from the same samples.

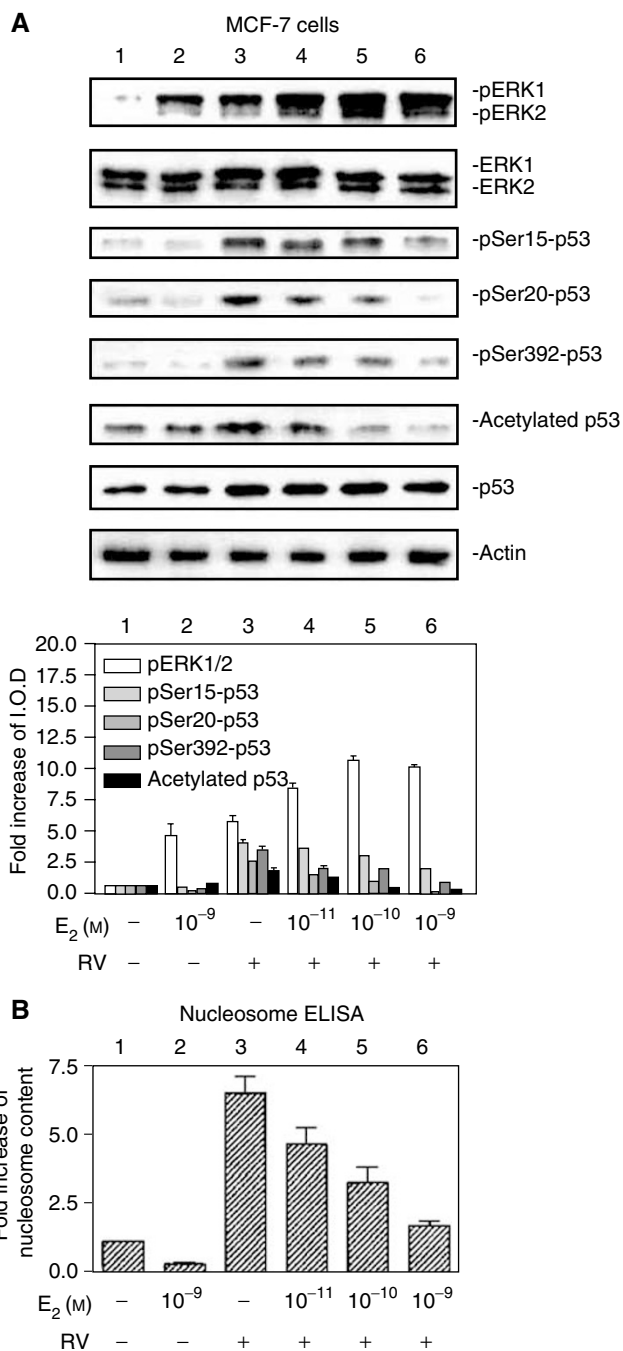


Figure 4 E₂ inhibits resveratrol-induced p53 phosphorylation and acetylation, and subsequent apoptosis, in a concentration-dependent manner. **(A)** MCF-7 cells were treated with E₂ (10⁻¹¹–10⁻⁹ M) with or without resveratrol (RV, 10 μM) for 4 h. Immunoblots of nuclear fractions from this representative experiment show that resveratrol-induced MAPK activation (pERK1/2, top panel, lane 3) was enhanced by 10⁻¹¹–10⁻⁹ M E₂ (lanes 3–6; *P*<0.005). However, resveratrol-induced ser15, ser20 and ser392 phosphorylation of p53, as well as acetylation of p53, was inhibited by E₂ in a dose-dependent manner (*P*<0.001, lanes 3–6 for all these parameters), with the most marked inhibition occurring at 10⁻⁹ M E₂ (lane 6). Molecular weight markers in this figure are not shown, but are similar to those shown in Figure 1. **(B)** This graph shows the extent of apoptosis, measured by nucleosome ELISA, in MCF-7 cells treated with E₂ (10⁻¹¹–10⁻⁹ M) with or without resveratrol (RV, 10 μM) for 24 h. Resveratrol alone caused apoptosis (lane 3, *P*<0.001). E₂ alone did not induce apoptosis, but the hormone inhibited nucleosome formation induced by resveratrol in a dose-dependent manner (*P*<0.001). Means ± s.e.m. of five experiments are shown.

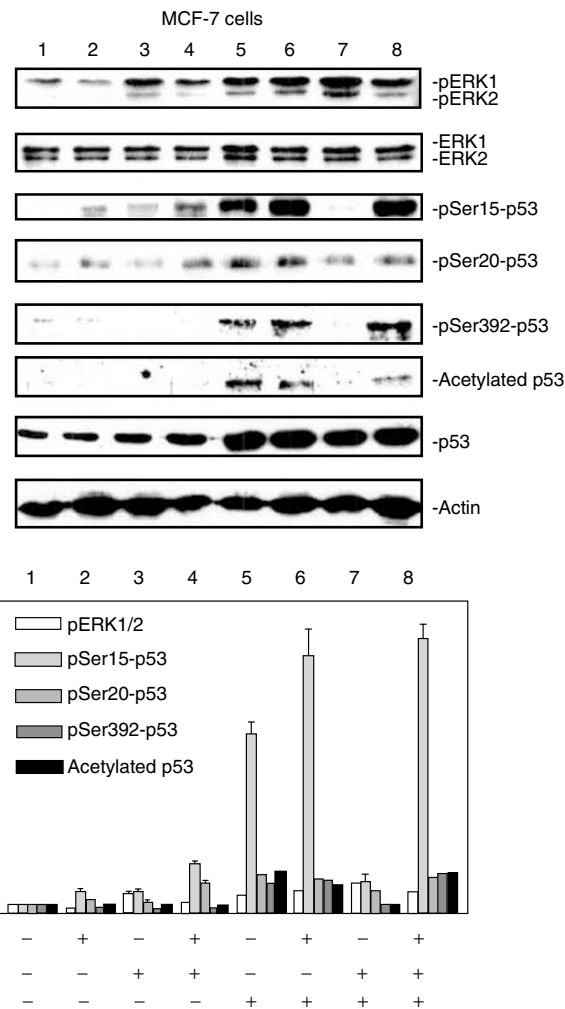


Figure 5 ICI 182,780 (ICI) inhibits the effects of E₂ on resveratrol-induced activation of MAPK and p53. MCF-7 cells were pretreated with 3 nM ICI or diluent for 30 min, after which they were treated with 10⁻⁹ M E₂ and/or 10 μM resveratrol for 4 h along with continued 3 nM ICI or diluent as in the pretreatment period. Results shown are representative of three experiments. Immunoblots of nuclear fractions show that ICI minimally inhibited E₂-induced MAPK activation (comparing lanes 3 and 4), but did not inhibit resveratrol-induced MAPK activation (lanes 5 and 6). ICI 182,780 did enhance resveratrol-induced ser15-p53 phosphorylation, although this effect was not statistically significant. The additive effect on MAPK activation of E₂ and resveratrol was reduced by ICI (*P*<0.05, lane 7 vs lane 8), and the inhibitory effect of E₂ on resveratrol-induced serine phosphorylation and acetylation of p53 was partially reversed by ICI treatment (*P*<0.02 for each parameter, comparing lanes 7 and 8). Molecular weight markers in this figure are not shown, but are similar to those shown in Figure 1.

Apoptosis/nucleosomes

Cells were harvested and washed twice with phosphate-buffered saline. Nucleosome ELISA assays were carried out according to the protocol provided by Oncogene Research Products (Cambridge, MA, USA) (Lin *et al*, 2002).

Apoptosis/DNA fragmentation

Total genomic DNA was extracted according to the protocol of the SUICIDE-TRACK™ DNA LADDER ISOLATION KIT (Oncogene, Cambridge, MA, USA). DNA electrophoresis was performed in 1.5% agarose gels containing 0.2 μg ml⁻¹ of ethidium bromide.

Gels were visualised under UV light and photographed with Polaroid film (Polaroid Co., Cambridge, MA, USA). Photographs were scanned under direct light (BioImage, Millipore) for quantitation and illustration.

RESULTS

Effects of resveratrol and a p53 inhibitor on MAPK activation, p53 phosphorylation and apoptosis in MCF-7 cells

Initial studies focused on the effects of resveratrol on phosphorylation, or activation, of MAPK (pERK1/2) and p53, and accumulation of these phosphorylated proteins in nuclear fractions of MCF-7 cells (Figure 1). Treatment of these cells with 1–100 μM resveratrol for 4 h induced MAPK phosphorylation and serine phosphorylation of p53 at residues 15, 20 and 392. In addition, acetylation of p53 was induced by resveratrol treatment (Figure 1). Phosphorylation of ERK1 and ERK2, and of p53, resulted in translocation of these proteins to cell nuclei. The combined results from three experiments, normalised to a value of 1 in control samples, are shown in the graph of Figure 1.

In order to determine if resveratrol-induced apoptosis was p53 dependent, a specific p53 inhibitor, pifithrin- α (PFT- α) (Komarov *et al*, 1998), was added to cells in the presence or absence of resveratrol. Resveratrol-induced phosphorylation of serines 15, 20 and 392, and acetylation of p53, were inhibited by PFT- α (Figure 2A). In Figure 2B, the combined results of three nucleosome ELISA studies show a five-fold increase in nucleosome content in cells treated with resveratrol, and a reduction in the resveratrol effect with PFT- α . Increased nucleosome content is directly related to apoptosis (Lin *et al*, 2002; Shih *et al*, 2002). These findings indicate that resveratrol-induced apoptosis in MCF-7 cells is p53-dependent.

Effect of oestradiol on resveratrol-induced MAPK activation, serine phosphorylation of p53 and apoptosis

In a 4 h study with MCF-7 cells, E_2 (10^{-9}M) caused phosphorylation (activation) and nuclear translocation of MAPK, shown as pERK1 and pERK2 in Figure 3, lane 2. Furthermore, in cells treated with 10 μM resveratrol for 4 h, 1–4 h of E_2 exposure enhanced the activation and nuclear translocation of MAPK caused by resveratrol (lanes 4–7 compared with lane 3). In contrast to this enhancement by E_2 of resveratrol-induced MAPK activation, the hormone inhibited resveratrol-induced serine phosphorylation of p53 in a time-dependent manner. Phosphorylation of serines 15, 20 and 392 by resveratrol, as well as acetylation of p53, were all progressively reduced in resveratrol-treated MCF-7 cells by the addition of E_2 to resveratrol for 1–4 h.

In E_2 concentration–response studies, there was hormone concentration-dependent enhancement of resveratrol-stimulated MAPK activation with 10^{-11} – 10^{-9}M E_2 (Figure 4A, lanes 4–6) but reduction in resveratrol-induced phosphorylation of p53 serines 15, 20 and 392, and reduction in acetylation of p53 after treatment with both agents for 4 h (lanes 4–6). These latter effects were also E_2 concentration-dependent. The effect of 10^{-11} – 10^{-9}M E_2 on resveratrol-induced apoptosis in MCF-7 cells, indicated by nucleosome ELISA, is shown in Figure 4B. Dose-responsive inhibition by E_2 of apoptosis in these resveratrol-treated cells correlates directly with the reduction in post-translational modification of p53 seen in Figure 4A, above.

Effect of the oestrogen receptor- α (ER) inhibitor, ICI 182,780, on actions of resveratrol and oestradiol

E_2 has been shown to block chemotherapy- or radiation-induced apoptosis through a plasma membrane receptor that appears to be

the nuclear ER located in the cell membrane (Razandi *et al*, 2000). Therefore, we tested the E_2 effect on resveratrol action in the presence of ICI 182,780 (ICI), an inhibitor of ER (Papaconstantinou *et al*, 2002). ICI 182,780 partially suppressed E_2 -induced ERK1/2 phosphorylation, or activation (Figure 5, comparing lanes 3 and 4). ICI 182,780 did not inhibit resveratrol-induced post-translational modifications of p53 (lane 5 vs lane 6). ICI 182,780 did enhance the effect of resveratrol on serine-15 phosphorylation of p53 (lane 6), although this effect was not statistically significant. E_2 blocked serine phosphorylation and acetylation of p53 caused by resveratrol (lane 7), but in the presence of E_2 and ICI, the inhibitory effect of the hormone on phosphorylation and acetylation of p53 was not seen (lane 8). In summary, signal transduction by resveratrol was enhanced by ICI, and the action on MAPK activation by E_2 alone was inhibited by ICI. The inhibitory effect of E_2 on resveratrol-induced phosphorylation and acetylation of p53 appears to be ER-mediated, in that this hormone effect was reversed by the ER inhibitor.

Oestradiol inhibits resveratrol-induced p53 binding to DNA and p53-dependent gene expression

We studied the interaction of p53 with a p53-binding radiolabelled oligonucleotide added to nuclear extracts from resveratrol-treated cells, in the presence or absence of E_2 . Results shown in Figure 6 indicate that (1) resveratrol caused binding of p53 to the labelled oligonucleotide, and (2) E_2 inhibited interaction of p53 and the oligonucleotide. Therefore, E_2 not only inhibits resveratrol-induced post-translational modification of p53 as shown above in Figures 3–5 but also inhibits DNA binding by p53.

Resveratrol is known to induce p53-dependent *p21* expression in human thyroid cancer cells (Shih *et al*, 2002), an effect that is associated with apoptosis in these cells. Induction by resveratrol of *p21* expression in MCF-7 cells has been previously shown to lead to apoptosis (Poza-Guisado *et al*, 2002). In our studies of transcription by RT–PCR in MCF-7 cells, we found that resveratrol caused transcription of *p21*, and that E_2 inhibited this action of the

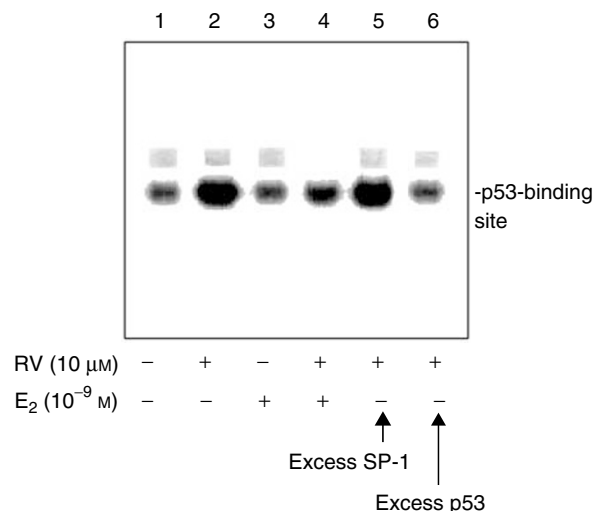


Figure 6 E_2 inhibits resveratrol-induced p53 binding to a relevant oligonucleotide in an EMSA. The assay was carried out with nuclear fractions prepared after 4 h treatment of cells with 10 μM resveratrol, 10^{-9}M E_2 , or both agents. p53 binding to the radiolabelled oligonucleotide was seen (lane 2). E_2 inhibited p53 binding (lane 4 compared with lane 2). Excess unlabelled oligonucleotide (p53-oligo) decreased p53 binding to the labelled form (lane 6), whereas there was no change in p53 binding with an excess of unlabelled nonspecific SP-1 oligonucleotide (lane 5). This figure is representative of three similar experiments.

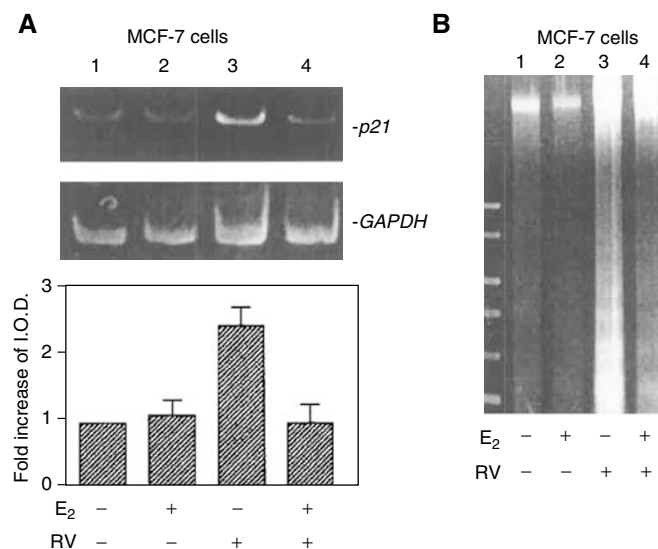


Figure 7 E₂ inhibits p53-inducible *p21* expression and apoptosis caused by resveratrol. **(A)** MCF-7 cells were treated with 10 μ M resveratrol in the presence or absence of 10⁻⁹ M E₂ for 24 h. Based on measurement of *p21* and *GAPDH* cDNA band densities (upper and lower images, respectively), and correction of *p21* densities for the levels of *GAPDH*, a 2.5-fold increase in *p21* cDNA abundance was seen with resveratrol treatment (lane 3 compared with lane 1). This increase in *p21* expression was significant ($P < 0.025$) by analysis of variance. E₂, 10⁻⁹ M, blocked *p21* transcription induced by resveratrol (lane 4 compared with lane 3, $P < 0.05$), but had no effect in the absence of resveratrol. **(B)** MCF-7 cells were treated with 10 μ M resveratrol in the presence or absence of 10⁻⁹ M E₂ for 24 h. Resveratrol caused DNA fragmentation, indicating apoptosis as shown in this representative figure (lane 3). This effect of resveratrol was inhibited by E₂ (lane 4).

stilbene (Figure 7A). The biological end point of this effect of E₂ was inhibition of resveratrol-induced apoptosis, as indicated by loss of DNA fragmentation (Figure 7B) and nucleosome ELISA (Figure 4B).

Oestradiol, 10⁻⁹ M, had no effect alone on *p21* expression in our experiments. Cariou *et al* (2000) have described a transient increase in *p21* content after 4 h treatment with 10⁻⁸ M E₂ in MCF-7 cells, whereas in our studies, a lower concentration of hormone was used for 24 h. Others have reported that E₂ can enhance abundance of *p21* mRNA in D351 breast cancer cells (Levenson *et al*, 2003). The basis for these disparate effects of E₂ is not clear, although differences in cell lines, in hormone concentration or in exposure time of cells to E₂ (Levenson *et al*, 2003) may have contributed to the different results. The D351 cells used by Levenson and co-workers were MDA-MB-231 cells transfected with cDNA for *wt* ER, but the p53 in these cells is said to be nonfunctional (Toillon *et al*, 2002).

DISCUSSION

Resveratrol, a phyto-oestrogen based on the fact that it binds to and activates ER, has similar affinity for ER α and ER β , but with 7000-fold lower affinity than E₂ (Bowers *et al*, 2000). As has been shown for other selective ER modulators such as tamoxifen, resveratrol has been considered to have potential as an anti-breast cancer adjunct (Bhat *et al*, 2001). The stilbene has been shown to inhibit carcinogen-induced preneoplastic lesions and mammary tumours in rodent models (Bhat *et al*, 2001). In the present studies, treatment of MCF-7 breast cancer cells with 1–100 μ M resveratrol induced MAPK activation, serine phosphorylation and acetylation of p53 and p53-dependent apoptosis. In addition to these findings

in breast cancer cells, we have previously shown that human thyroid and prostate cancer cells are susceptible to resveratrol-induced, p53-dependent apoptosis (Lin *et al*, 2002; Shih *et al*, 2002).

We have examined the possibility that the actions of resveratrol on MCF-7 cells might be modified by E₂. Acting at the cell surface, oestradiol has been shown by several investigators to rapidly activate MAPK (Migliaccio *et al*, 1996; Razandi *et al*, 2003; Wade and Dorsa, 2003). We have confirmed this action of E₂ in MCF-7 cells in this report (Figures 3–5). Mitogen-activated protein kinase activation induced by oestrogen in breast cancer cells has been linked to cell proliferation but there have been no reports to indicate that MAPK activation in breast cancer cells is involved in apoptosis (Santen *et al*, 2002). In our studies, treatment of MCF-7 cells with 10 μ M resveratrol in the presence of 10⁻⁹ M E₂ produced additive MAPK activation by E₂ and resveratrol. However, E₂ inhibited resveratrol-induced phosphorylation of serines 6, 15 and 392 in p53, as well as acetylation of p53, despite the enhancement by E₂ of MAPK activity.

A downstream effect of this action of oestrogen on MAPK activation may be phosphorylation of Ser-118 of ER α and enhanced transcriptional activity of the receptor (Kato *et al*, 1995). We did not observe apoptosis as a downstream consequence of MAPK activation by E₂ alone, compared with the actions of resveratrol. Thus, the confluence at MAPK of signals generated by resveratrol and E₂ results, downstream of MAPK, in a divergence of effects on nuclear transcription by p53 and by E₂. This is consistent with the existence of discrete intracellular pools of MAPK (ERK1/2) separately regulated by resveratrol and E₂.

The antiapoptotic effect of E₂ in the presence of resveratrol involves rapid stimulation of signalling cascades, including the ERK1/2 pathway. In our studies, the ER inhibitor ICI 182,780 suppressed both E₂-induced MAPK activation and the inhibitory effect of E₂ on resveratrol-induced serine phosphorylation of p53. Although resveratrol has been shown by others to have oestrogen-like activity in a transcriptional assay in cells expressing predominantly ER α (Bhat *et al*, 2001), we found that ICI 182,780, in a relatively low concentration of 3 nM, did not diminish resveratrol-induced activation of MAPK and serine phosphorylation of p53, and in fact appeared to enhance these effects of the stilbene. The concentration we used was similar to the inhibitory 10 nM concentration used in MCF-7 cells by Cariou *et al* (2000).

Others have also reported that activities of resveratrol are not affected by ICI 182,780 (Cho *et al*, 2002). Since E₂ action was inhibited by ICI 182,780 in our studies, while resveratrol activity was minimally affected by ICI 182,780, the inhibitory effect of E₂ on resveratrol-induced serine phosphorylation of p53 was interpreted to be ER mediated. Studies by others have shown that ICI 182,780 may enhance the effects of doxorubicin, paclitaxol and tumour necrosis factor- α on the production of apoptosis in MCF-7 cells (Burow *et al*, 2001).

Oestradiol alone had no effect on the binding of p53 to a relevant oligonucleotide. However, E₂ did inhibit resveratrol-induced binding of p53 to the oligonucleotide (Figure 6). This finding is consistent with E₂ inhibition of post-translational modifications of p53 that are essential for p53-dependent gene transcription. These results have implications for the possible use of resveratrol therapeutically in cancer treatment, as they suggest that resveratrol may be most effective in an oestrogen-depleted environment.

The transcriptional activity of p53 is subject to modulation by other hormones as well. For example, glucocorticoids prevent p53-induced apoptosis through interaction of the glucocorticoid receptor (GR) with p53 and the E3 ubiquitin ligase, Hdm2 (Sengupta and Wasyluk, 2001) in cytosol. Another example of a modulator is the *mdm2* gene that is positively regulated by p53, yet the *mdm2* protein binds p53, forming a feedback loop that controls *mdm2* activity (Qi *et al*, 1999).

Since the effect of resveratrol that we have demonstrated in MCF-7 cells centres on the modification and action of the tumour suppressor p53, it is conceivable that cancer cells harbouring mutant p53 might not respond in the same manner. However, studies by Hsieh *et al* (1999) have documented resveratrol-induced reduction in growth of MDA-MB-435 cells, which contain a mutant p53. In our studies of the actions of resveratrol on DU145 prostate cancer cells, which also contain mutant p53 (Zhang *et al*, 2003), resveratrol did induce apoptosis. Future studies are to be directed towards the identification of critical sites on p53 for resveratrol action.

The present studies indicate that ambient E₂ may antagonise actions of resveratrol in selected treatment paradigms. Our results

suggest the complexity of actions of resveratrol in both *in vivo* and *in vitro* settings. Were resveratrol to be tested in intact animals, it would be important to control for levels of oestrogen, and useful to examine actions of the stilbene in the presence of an E₂ antagonist.

ACKNOWLEDGEMENTS

This work has been supported by grants from the Research Service, United States Department of Veterans Affairs, and from the Charitable Leadership, Candace King Weir and Beltrone Foundations.

REFERENCES

- Agarwal ML, Taylor WR, Chernov MV, Chernova OB, Stark GR (1998) The p53 network. *J Biol Chem* **273**: 1–4
- Ashcroft M, Vousden KH (1999) Regulation of p53 stability. *Oncogene* **18**: 7637–7643
- Berlev NA, Liu L, Chehab NH, Mansfield K, Harris KG, Halazonetis TD, Berger SL (2001) Acetylation of p53 activates transcription through recruitment of coactivators/histone acetyltransferases. *Mol Cell* **8**: 1243–1254
- Bhat KP, Lantvit D, Christov K, Mehta RG, Moon RC, Pezzuto JM (2001) Estrogenic and antiestrogenic properties of resveratrol in mammary tumor models. *Cancer Res* **61**: 7456–7463
- Bowers JL, Tyulmenkov VV, Jernigan SC, Klinge CM (2000) Resveratrol acts as a mixed agonist/antagonist for estrogen receptors α and β . *Endocrinology* **141**: 3657–3667
- Burow ME, Weldon CB, Tang Y, McLachlan JA, Beckman BS (2001) Oestrogen-mediated suppression of tumour necrosis factor α -induced apoptosis in MCF-7 cells: subversion of Bcl-2 by anti-oestrogens. *J Steroid Biochem Mol Biol* **78**: 409–418
- Cariou S, Donovan JC, Flanagan WM, Milic A, Bhattacharya N, Slingerland JM (2000) Down-regulation of p21WAF1/CIP1 or p27Kip1 abrogates antiestrogen-mediated cell cycle arrest in human breast cancer cells. *Proc Natl Acad Sci USA* **97**: 9042–9046
- Cho DI, Koo NY, Chung WJ, Kim TS, Ryu SY, Im SY, Kim KM (2002) Effects of resveratrol-related hydroxystilbenes on the nitric oxide production in macrophage cells: structural requirements and mechanism of action. *Life Sci* **71**: 2071–2082
- Clement M-V, Hirpara JL, Chawdhury S-H, Pervaiz S (1998) Chemopreventive agent resveratrol, a natural product derived from grapes, triggers CD95 signaling-dependent apoptosis in human tumor cells. *Blood* **92**: 996–1002
- Higashimoto Y, Saito S, Tong XH, Hong A, Sakaguchi K, Appella E, Anderson CW (2000) Human p53 is phosphorylated on serines 6 and 9 in response to DNA damage-inducing agents. *J Biol Chem* **275**: 23199–23203
- Hsieh TC, Burfeind P, Laud K, Backer JM, Traganos F, Darzynkiewicz Z, Wu JM (1999) Cell cycle effects and control of gene expression by resveratrol in human breast cancer cell lines with different metastatic potentials. *Int J Oncol* **15**: 245–252
- Huang C, Ma WY, Goranson A, Dong Z (1999) Resveratrol suppresses cell transformation and induces apoptosis through a p53-dependent pathway. *Carcinogenesis* **20**: 237–242
- Jang M, Cai L, Udeani GO, Slowing KV, Thomas CF, Beecher CW, Fong HH, Farnsworth NR, Kinghorn AD, Mehta RG, Moon RC, Pezzuto JM (1997) Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. *Science* **275**: 218–220
- Jeanet P, Bessis R, Gautheron B (1991) The production of resveratrol (3,5,4'-trihydroxystilbene) by grape berries in different stages. *Am J Enol Viticul* **42**: 41–46
- Kato S, Endoh H, Masuhiro Y, Kitamoto T, Uchiyama S, Susaki H, Masushige S, Gotoh Y, Nishida E, Kawashima H, Metzger D, Chambon P (1995) Activation of the estrogen receptor through phosphorylation by mitogen-activated protein kinase. *Science* **270**: 491–494
- Komarov PG, Komarova EA, Kondratov RV, Christov-Tselkov K, Coon JS, Chernov MV, Gudkov AV (1998) A chemical inhibitor of p53 that protects mice from the side effects of cancer therapy. *Science* **285**: 1733–1737
- Levenson AS, Gehm BD, Pearce ST, Horiguchi J, Simons LA, Ward III JE, Jameson JL, Jordan VC (2003) Resveratrol acts as an estrogen receptor (ER) agonist in breast cancer cells stably transfected with ER α . *Int J Cancer* **104**: 587–596
- Lin H-Y, Shih A, Davis FB, Tang H-Y, Martino LJ, Bennett JA, Davis PJ (2002) Resveratrol-induced serine phosphorylation of p53 causes apoptosis in a mutant p53 prostate cancer cell line. *J Urol* **168**: 748–755
- Liu L, Scolnick DM, Trievel RC, Zhang HB, Marmorstein R, Halazonetis TD, Berger SL (1999) p53 sites acetylated *in vitro* by PCAF and p300 are acetylated *in vivo* in response to DNA damage. *Mol Cell Biol* **19**: 1202–1209
- Lu R, Serrero G (1999) Resveratrol, a natural product derived from grape, exhibits antiestrogenic activity and inhibits the growth of human breast cancer cells. *J Cell Physiol* **179**: 297–304
- Migliaccio A, Di Domenico M, Castoria G, de Falco A, Bontempo P, Nola E (1996) Tyrosine kinase/p21ras/MAP-kinase pathway activation by estradiol-receptor complex in MCF-7 cells. *EMBO J* **15**: 1292–1300
- Pace-Asciak C, Hahn R, Diamandis EP, Soleas G, Goldberg DM (1995) The red wine phenolics trans-resveratrol and quercetin block human platelet aggregation and eicosanoid synthesis: Implications for protection against coronary disease. *Clin Chim Acta* **235**: 207–219
- Papaconstantinou AD, Umbreit TH, Goering PL, Brown KM (2002) Effects of 17 α -methyltestosterone on uterine morphology and heat shock protein expression are mediated through estrogen and androgen receptors. *J Steroid Biochem Mol Biol* **82**: 305–314
- Pozo-Guisado E, Alvarez-Barrientos A, Mulero-Navarro S, Santiago-Josefat B, Fernandez-Salguero PM (2002) The antiproliferative activity of resveratrol results in apoptosis in MCF-7 but not in MDA-MB-231 human breast cancer cells: cell-specific alteration of the cell cycle. *Biochem Pharmacol* **64**: 1375–1386
- Prives C, Manley JL (2001) Why is p53 acetylated? *Cell* **107**: 815–818
- Qi JS, Yuan Y, Desai-Yajnik V, Samuels HH (1999) Regulation of the mdm2 oncogene by thyroid hormone receptor. *Mol Cell Biol* **19**: 864–872
- Razandi M, Alton G, Pedram A, Ghonshani S, Webb P, Levin ER (2003) Identification of a structural determinant necessary for the localization and function of estrogen receptor α at the plasma membrane. *Mol Cell Biol* **23**: 1633–1646
- Razandi M, Pedram A, Levin ER (2000) Plasma membrane estrogen receptors signal to antiapoptosis in breast cancer. *Mol Endocrinol* **14**: 1434–1447
- Sakaguchi K, Herrera JE, Saito S, Miki T, Bustin M, Vassilev A, Anderson CW, Appella E (1998) DNA damage activates p53 through a phosphorylation-acetylation cascade. *Genes Dev* **12**: 2831–2841
- Santen RJ, Song RX, McPherson R, Kumar R, Adam L, Jeng MH, Yue W (2002) The role of mitogen-activated protein (MAP) kinase in breast cancer. *J Steroid Biochem Mol Biol* **80**: 239–256
- Sengupta S, Wasylyk B (2001) Ligand-dependent interaction of the glucocorticoid receptor with p53 enhances their degradation by Hdm2. *Genes Dev* **15**: 2367–2380
- She QB, Bode AM, Ma WY, Chen NY, Dong Z (2001) Resveratrol-induced activation of p53 and apoptosis is mediated by extracellular-signal-regulated protein kinases and p38 kinase. *Cancer Res* **61**: 1604–1610

- Shih A, Davis FB, Lin H-Y, Davis PJ (2002) Resveratrol induces apoptosis in thyroid cancer cell lines via a mitogen-activated protein kinase- and p53-dependent mechanism. *J Clin Endo Metab* **87**: 1223–1232
- Shih A, Lin HY, Davis FB, Davis PJ (2001) Thyroid hormone promotes serine phosphorylation of p53 by mitogen-activated protein kinase. *Biochemistry* **40**: 2870–2878
- Toillon RA, Chopin V, Jouy N, Fauquette W, Boilly B, Le Bourbis X (2002) Normal breast epithelial cells induce p53-dependent apoptosis and p53-independent cell cycle arrest of breast cancer cells. *Breast Cancer Res Treat* **71**: 269–280
- Wade CB, Dorsa DM (2003) Estrogen activation of cyclic adenosine 5'-monophosphate response element-mediated transcription requires the extracellularly regulated kinase/mitogen-activated protein kinase pathway. *Endocrinology* **144**: 832–838
- Zhang Z, Li M, Wang H, Agrawal S, Zhang R (2003) Antisense therapy targeting MDM2 oncogene in prostate cancer: effects on proliferation, apoptosis, multiple gene expression, and chemotherapy. *Proc Natl Acad Sci USA* **100**: 11636–11641