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Resveratrol, but not dihydroresveratrol, induces premature senescence in primary human fibroblasts

Richard G. A. Faragher · Dominic G. A. Burton · Patricia Majecha · Noel S. Y. Fong · Terence Davis · Angela Sheerin · Elizabeth L. Ostler

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Abstract Resveratrol, *trans*-3,5,4′-trihydroxystilbene, is a polyphenolic compound which has been reported to mimic the gene expression patterns seen in whole animals undergoing dietary restriction. The mechanism of action of resveratrol remains poorly understood, but modulation of both cellular proliferation and apoptosis has been proposed as important routes by which the molecule may exert its effects. This study reports the effects of both resveratrol and dihydroresveratrol (a primary in vivo metabolite) on the proliferative capacity of human primary fibroblasts. No generalised reduction in the growth fraction was observed when fibroblasts derived from three different tissues were treated with resveratrol at concentrations of 10 μm or less. However, concentrations above 25 μm produced a dose-dependent reduction in proliferation. This loss of the growth fraction was paralleled by an increase in the senescent fraction as determined by staining for senescence associated beta galactosidase and dose recovery studies conducted over a 7-day period. Entry into senescence in response to treatment with resveratrol could be blocked by a 30-min preincubation with the p38 MAP kinase inhibitor SB203580. No effects on proliferation were observed when cells were treated with dihydroresveratrol at concentrations of up to 100 μm.

Keywords Resveratrol · Cellular senescence · Ageing · MRC5 · HCA2 · Ek1.Br · p38 MAP kinase

R. G. A. Faragher (✉) · P. Majecha · N. S. Y. Fong · A. Sheerin · E. L. Ostler
School of Pharmacy and Biomolecular Sciences,
Cockcroft Building, University of Brighton,
Lewes Road, Moulsecoomb,
Brighton, East Sussex, UK
e-mail: rgaf@bton.ac.uk

D. G. A. Burton
Division of Gerontology and Geriatric Medicine,
Department of Medicine,
University of Miami, Miller School of Medicine,
Miami, FL, USA

T. Davis
Department of Pathology, School of Medicine,
University of Cardiff,
Heath Park,
Cardiff, Wales, UK

Introduction

The putative anti-ageing properties of resveratrol (*trans*-3,5,4′, trihydroxystilbene) are currently attracting considerable interest within the research community. Resveratrol is a phytoalexin found in high concentrations in grapes and a variety of other plant materials (Pascual-Martí et al. 2001). It has been reported (Pearson et al. 2008, Barger et al. 2008) that supplementation of the standard diet of laboratory mice with resveratrol induces a shift in patterns of gene expression in multiple tissues that is similar to that seen when animals are subjected to dietary restriction (DR). Thus, it has been proposed that resveratrol acts

in some contexts as a DR mimetic, though consistent evidence of lifespan extension using the molecule is currently lacking (Wood et al. 2004, Howitz et al. 2003, Valenzano et al. 2006, Bass et al. 2007, Pearson et al. 2008). The effects of resveratrol have been intensively studied since its "anti-ageing" activity was first reported. However, a consensus has yet to emerge regarding either the primary mode of action of the compound or its utility.

On the basis of both in vitro and in vivo studies, resveratrol has been reported to display a wide range of activities that could potentially assist in the extension of healthy life (reviewed in Saiko et al. 2008). The molecule can be used to block peroxide-induced damage and has been shown to be neuroprotective. It can trigger cytostasis in models of cancer, induces enzymes involved in xenobiotic metabolism and increases SirT1 activity. Unsurprisingly, many of the reported activities of resveratrol have been shown to be both concentration-dependent and cell type-specific.

Although a consensus has yet to emerge regarding which activities of resveratrol are most important, alteration of the rate of cell turnover is frequently implicated as a primary mechanism by which the molecule produces beneficial effects in mammals. The anticancer activity of resveratrol is a case in point. A reduction in tumour formation would be expected to enhance organismal lifespan and multiple tumour cell lines treated with micromolar concentrations of resveratrol respond by either apoptosis or entry into a variety of cytostatic states (Jang et al. 1997, Rusin et al. 2009). However, mechanisms which ascribe a central role to the antioxidant properties of the molecule also invoke cell turnover, albeit indirectly, (since reduced levels of tissue damage would be predicted to reduce the burden on cellular repair and replacement processes).

The activity of resveratrol has been heavily studied in vivo using tumour-prone rodents and in vitro in cancer cell lines but very little work has been conducted to date on primary human cell strains. This lack of data represents a significant gap in our understanding of the anti-cancer potential of resveratrol and related molecules since selectivity against tumour cells is a highly desirable property for therapeutic compounds. Recently, we demonstrated that cultures of human vascular smooth muscle cells treated with resveratrol showed a dose-dependent decrease in their growth fraction, accompanied by a dose-dependent increase in DNA damage (Burton et al. 2007). Interestingly, markers of apoptosis or replicative senescence were not induced indicating that (in this cell type) resveratrol appears to induce a relatively benign cytostatic state rather than a senescent one. Studies of this type are important if a better understanding of the likely effects of resveratrol in a human context is to be obtained.

In vivo, dietary resveratrol crosses the mammalian gut well but is rapidly metabolised and thus shows very limited subsequent bioavailability in the majority of tissues. Almost no unmetabolised resveratrol is observed in human plasma, but micromolar concentrations of glucuronidated and sulphated analogues can be detected, alongside the reduced metabolite, dihydroresveratrol (Walle et al. 2004). The effects of these metabolites have been studied to a much more limited extent than the parent compound but are of considerable relevance to researchers interested in the in vivo use of resveratrol and its analogues. Accordingly, this paper reports the effects of treatment of primary human fibroblasts with both resveratrol and dihydroresveratrol.

Methods

Materials

Resveratrol, dimethyl sulfoxide and 5% palladium on carbon were obtained from Sigma-Aldrich. Acetone, methanol, hexane and ethyl acetate were obtained from Fisher Scientific. Dichloromethane and celite were obtained from Acros Organics. The primary antibody used for immunofluorescence was anti-Ki-67 and the secondary antibody polyclonal rabbit anti-mouse immunoglobulin/FITC was obtained from Dako Cytomation. Vectashield mountant, Hematoxylin QS and Nuclear Fast Red were obtained from Vector Laboratories, the foetal calf serum (FCS), non-essential amino acids, modified Eagle's medium, penicillin and streptomycin were obtained from PAA Laboratory. The human diploid foetal lung fibroblasts (MRC5) were obtained from the Coriell institute for medical research (AG05965B). All materials were used as supplied. The identity, purity and stability (>7 days,

no discernable change in spectra) in dimethyl sulphoxide of resveratrol were confirmed by proton nuclear magnetic resonance and high resolution mass spectrometry.

Methods

Synthesis of α, β-dihydroresveratrol

The synthesis of α, β-dihydroresveratrol was based on the methods of Stivala et al. (2001) and is described below. α,β-Dihydro-3,4,5-trihydroxystilbene (α,β-dihydroresveratrol) was obtained by catalytic hydrogenation of *trans*-resveratrol (150 mg, 0.66 mmol) with 10% Pd/C catalyst in methanol (5 ml) at room temperature under atmospheric pressure. The catalyst was removed by filtration through celite and the filtrate evaporated to give a solid residue which was purified over silica gel (7:3, hexane/ethyl acetate) to obtain a brown sticky powder (97 mg, 0.42moles, 64%). This was recrystallised from ethyl acetate/hexane to give a pale brown solid. This was confirmed to be dihydroresveratrol by ^{1}H NMR and IR. Purity was >95% by TLC. The primary data are given below.

360 MHz ^{1}H NMR (CD_3 OD): δ=6.99 (d, 2 H, J= 8.28 Hz, H3 and H5), 6.65 (d, 2 H, J=8.64 Hz, H2 and H6), 6.05 (s, 2 H, H2′ and H6′), 6.01 (s, 1 H, H4′), 2.61 (ddt, 4 H, CH_2CH_2) ppm. *m*/*z*=213.1 ($C_{14}H_{14}O_3$). IR (Nujol): υmax=3,254 cm^{-1}, 1,599, 1,512, 1,330, 1,226, 1,151, 1,101, 975, 820. MPt: 138–140°C (No Literature MPt available).

Thin layer chromatography (Silica Gel, 1:1 hexane: ethyl acetate) R_f=0.8 (resveratrol R_f=0.45)

Stock solutions of dihydroresveratrol were prepared in 1% DMSO and PBS and used on the same day.

Culture of human diploid foetal lung fibroblasts (MRC5, HCA2, Ek1.Br)

Human diploid foetal lung fibroblasts (MRC5) were obtained from the Coriell Institute for Medical Research (AG05965B) and grown at a seeding density of 1.3×10^4 cells/cm^2 in Modified eagle's medium with essential and non essential amino acids, 15% FCS, 2 mM glutamine, penicillin (50 U/L), streptomycin (50 μg/L). Cells were sub-cultured every 5–10 days by standard trypsin dispersion and viable cell numbers determined by trypan blue staining. Replicative lifespan was measured in population doubling calculated using the standard formula. The fibroblasts were grown on 13 mm diameter glass coverslips at identical seeding density to the parent culture. HCA2 neonatal foreskin fibroblasts (HCA2, a gift of Jim Smith, Houston, TX, USA) and Ek1.Br human ocular keratocytes (Kipling et al. 2009) were cultured under identical conditions.

Where applicable, a 30-min pretreatment step with 10 μM SB203580 was included. Resveratrol (0–100 μM) or dihydroresveratrol (25–100 μM) in DMSO (Sigma) were then added for 48 h. A vehicle-only control was also included.

Determination of proliferating fraction by Ki67 staining

The proliferating fraction by Ki67 staining was determined as previously described (Burton et al. 2007). MRC5 cells on coverslips were washed with PBS and fixed in a 1:1 mixture of methanol and acetone for 5–10 min at room temperature. The fixed cells were incubated for 1 h at room temperature with a primary antibody raised against Ki67 (Dako M0722, mouse anti-Ki67, diluted in 1% (*v*/*v*) FCS in PBS, 1/20 dilution). The cells were then washed three times in PBS and incubated for a further hour in the dark at room temperature with a secondary antibody conjugated with FITC (Dako F0261, rabbit anti-mouse FITC, diluted using 1% (*v*/*v*) FCS in PBS, 1/20 dilution). Coverslips were washed three times in PBS and once in water to remove excess salts. They were then mounted in a DAPI-containing mountant (Vectashield) and viewed on a fluorescent microscope. Cells traversing the cell cycle showed a distinctive pattern of nuclear staining. To determine the proliferating fraction, either 400 Ki67 positive or 1,000 total nuclei were counted in random fields on each of three coverslips.

Determination of senescence-associated β-galactosidase-positive fraction

MRC5 cells on cover slips were washed and fixed in 3% formaldehyde for 10 min at room temperature. The cells were washed and then incubated overnight at 37°C in a solution of 4-chloro-5-bromo-3-indolyl-β-D-galactoside in citric acid phosphate buffer containing potassium ferrocyanide (5 mM) and potassium ferricyanide (5 mM). As a positive control for β-galactosidase activity, one set of coverslips was

incubated in buffer at pH 4.0. To demonstrate senescence-associated β-galactosidase, the incubation buffer pH was raised to pH 6.0. The cells were counterstained using hematoxylin QS and nuclear fast red and viewed using a light microscope. To determine the senescent fraction, 1,000 cells were counted in random fields on two separate coverslips and the positive fraction calculated.

Dose recovery study

MRC5 fibroblasts at 30 population doublings were cultured as described above and were then treated for 48 h with either 50 or 100 μM resveratrol. The growth fraction was determined by Ki67 staining was determined immediately post-treatment and the assay was repeated under subconfluent conditions 7 days after removal of the resveratrol.

Results

Resveratrol induces growth arrest and senescence in normal human fibroblasts

Treatment of three separate strains of human fibroblasts with doses of resveratrol below 25 μM produced no generalised reduction in growth fraction although treatment of Ek1.Br fibroblasts with 5 μM resveratrol produced a small but significant decline in growth (Fig. 1, $p=0.0285$). Interestingly, treatment with of MRC5 fibroblasts with 10 μM resveratrol increased proliferation when compared to the 2.5 μM dose ($p=0.033$) although proliferation was not statistically altered compared to control values.

Treatment of all three strains of fibroblasts with resveratrol for 48 h at concentrations of 25 μM or above significantly reduced the fraction of Ki67-positive nuclei. The treated fibroblasts were enlarged and flattened compared to their untreated counterparts and bore a strong similarity to cells in a state of replicative senescence. There was no detectable apoptosis in the cultures as measured by TUNEL staining (data not shown).

In a more detailed study of a single cell strain, MRC5 fibroblasts (at 30, 42 and 56 cumulative population doublings) were treated with 25–100 μM resveratrol for 48 h. Again, pronounced changes in cellular morphology were seen and were accompanied by a significant ($p<0.001$, one-way ANOVA) dose dependent decrease in the culture growth fraction as assessed by Ki67 labelling (Fig. 2). Over the same concentration range, resveratrol also induced a dose-dependent increase in the fraction of fibroblasts showing detectable senescence-associated β-galactosidase (SA-β-galactosidase) activity (Fig. 3). These data are consistent with the induction of a senescent state; however, to confirm that the growth arrest observed was permanent an additional dose recovery study was undertaken. Cultures of MRC5 fibroblasts at 30 population doublings were exposed to 50 and 100 μM resveratrol for 48 h and the growth fraction determined (Fig. 4). Resveratrol treatment significantly

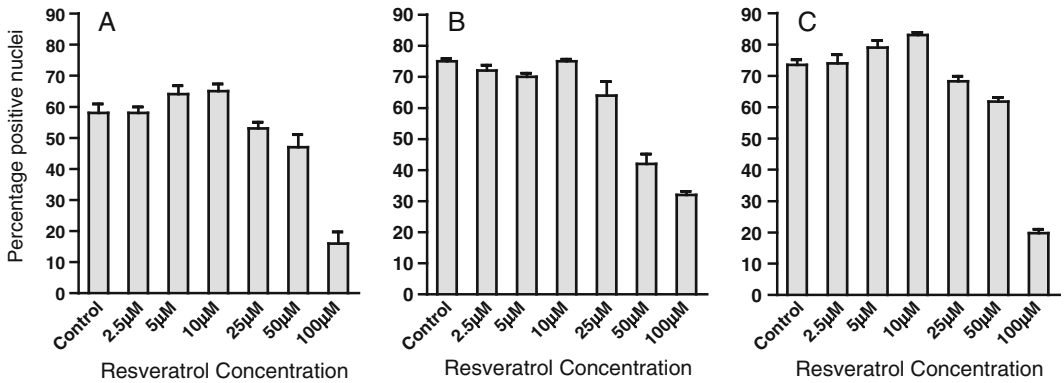

Fig. 1 Effects of resveratrol on the growth fraction of three different strains of primary human fibroblasts. **a** HCA2 fibroblasts at 36 population doublings, **b** EK1.Br fibroblasts at 44 population doublings and **c** MRC5 fibroblasts at 42 population doublings were exposed to a vehicle only control or to resveratrol over a dose range of 2.5–100 μM for 48 h. The *y*-axis show the percentage of Ki67-positive nuclei at each concentration for $n=3$ replicate cultures (±SEM). A statistically significant decrease in growth fraction was noted for EK1Br fibroblasts at 5 μM compared to vehicle only control ($p=0.0285$). Doses of 25 μM or above resulted in statistically significant declines in the growth fraction of all three cell strains

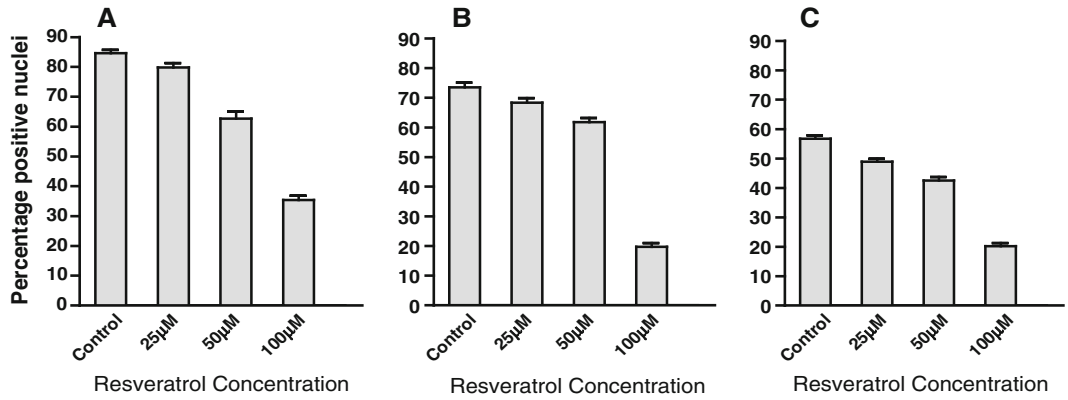

Fig. 2 Effects of resveratrol on the growth fraction of MRC5 fibroblasts. Replicate cultures (n=3) at **a** 30 population doublings, **b** 42 population doublings and **c** 56 population doublings were exposed to a vehicle only control or 25, 50 or 100 μM resveratrol for 48 h. The y-axes shows the percentage of Ki67 positive nuclei at each concentration and population doubling level (±SEM). All concentrations show a significant reduction in growth fraction compared to control (p<0.05). Overall, treated and non-treated cultures differ highly significantly (p<0.001)

reduced the fraction of positive nuclei (p=0.0149 at 50 μM and p=0.006 at 100 μM) and the growth fraction remained suppressed after a 7-day recovery period post-removal of the compound. These data are consistent with permanent removal of a fraction of cells from the proliferative pool.

Resveratrol-induced senescence can be blocked by SB203580

On the basis of prior reports that resveratrol induces growth arrest in tumour cells in a p38MAP kinase-dependent manner, we hypothesised that the senescence observed in MRC5 fibroblasts might result from usage of the same pathway. To test this hypothesis, MRC5 cells were pretreated with the p38MAPK inhibitor SB203580 at a concentration of 10 μM for 30 min prior to a 48-h incubation with resveratrol (25–100 μM). As shown in Fig. 5, at resveratrol concentrations of 50 μM and above, pretreatment with SB203580 significantly attenuated both the loss of the growth fraction and the increase in the SA-β-galactosidase positive fraction (p<0.05).

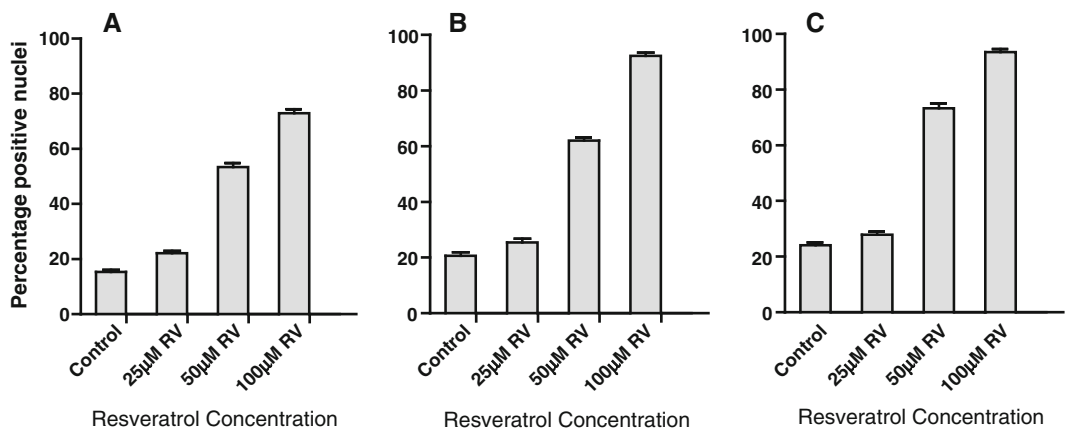

Fig. 3 Effects of resveratrol on the fraction of SA-β-galactosidase positive cells in MRC5 fibroblast cultures. Replicate cultures (n=3) at **a** 30 population doublings, **b** 42 population doublings and **c** 56 population doublings were exposed to a vehicle only control or 25, 50 or 100 μM resveratrol for 48 h. The y-axes show the percentage of SA-β-galactosidase positive cells at each concentration and population doubling level (±SEM). All treated populations show significantly increased numbers of positive cells relative to controls (p<0.05)

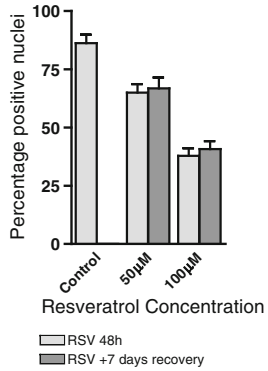

Fig. 4 Dose recovery study of MRC5 fibroblasts exposed to high concentrations of resveratrol. Cultures of MRC5 fibroblasts at 30 population doublings were exposed to either 50 or 100 μM resveratrol for 48 h and were then allowed to recover for a 7-day period after which the growth fraction was determined by Ki67 staining under subconfluent conditions. Resveratrol treatment significantly reduced the fraction of positive nuclei at both 50 (p=0.015) and 100 μM (p=0.006). However, there was no statistically significant difference in the growth fractions before and after the recovery period

Dihydroresveratrol does not induce senescence in MRC5 cells

In an identical manner to the experiments described above, MRC5 fibroblast cultures at three different population doubling levels were treated with 25–100 μM dihydroresveratrol for 48 h; after which the growth fraction was determined by Ki67 staining. The proliferating fraction of MRC5 cells after 48 h treatment with dihydroresveratrol did not differ significantly from vehicle only controls at any concentration assayed (Fig. 6).

Discussion and conclusions

Resveratrol has attracted considerable interest as an "anti-ageing" compound and has shown beneficial results in in vivo studies. The mechanisms by which resveratrol is proposed to exert its effects frequently invoke changes in the growth dynamics of cell populations. The most obvious case in point of this is when resveratrol is used as an anti-cancer agent. A very large body of in vitro data is available showing the growth suppressive effects of resveratrol on immortal cell lines. Unfortunately, very little data is available on the effects of the compound on primary cell strains such as MRC5 and HCA2 (normally considered a much better in vitro approximation to normal in vivo material). Such data are needed because there is very little evidence showing that resveratrol acts selectively against tumour cells (a highly desirable feature of chemotherapeutic agents). Ferry-Dumazet et al. (2002) showed that resveratrol decreased proliferation and induced apoptosis in cycling normal human peripheral blood lymphocytes at an IC_{50} <8 μM. However, in the same study, seven out of the eight leukemic cell lines tested had IC_{50} values for resveratrol which were significantly higher than this (~13 μM–43 μM). In contrast, Bellance et al. (2009) observed the selective induction of cell death in HLF-a epithelial carcinoma cells but not normal human fibroblasts when treated with either 50 or 200 μM resveratrol. However, Bellance et al. did not undertake a detailed characterization of the physiological state of the normal cells post-treatment with resveratrol at these doses.

In this study, we have shown that treatment of cultures of three different strains of fibroblasts with resveratrol over the concentration range commonly used in studies on tumour cell lines (25–100 μM) produces a dose-dependent decline in the growth fraction. It is also clear based on our dose recovery experiments that cells which exit the cell cycle as a result of treatment with 50 or 100 μM resveratrol are still non-dividing at least 7 days post-treatment. Given that the average cell cycle time of early passage human fibroblasts is typically less than 17–20 h (Grove and Cristofalo 1977), a failure of the proliferative fraction to recover after 168 h is compelling evidence for permanent arrest. Loss of the culture growth fraction is accompanied by a rapid increase in the fraction of cells displaying detectable SA-β-galactosidase activity compared to vehicle only controls. These phenotypic changes are the canonical features of cells which have entered replicative senescence.

It has been known for many years that there are subtle but significant differences in the cell cycle parameter of division competent cell populations at different population doubling levels (Grove and Cristofalo 1977). Accordingly, we tested the effects of resveratrol on populations of MRC5 human fibroblasts at 30, 42 and 56 population doublings. Induction of senescence occurs regardless of population doubling level and in each case the number of cells entering senescence can be substantially reduced

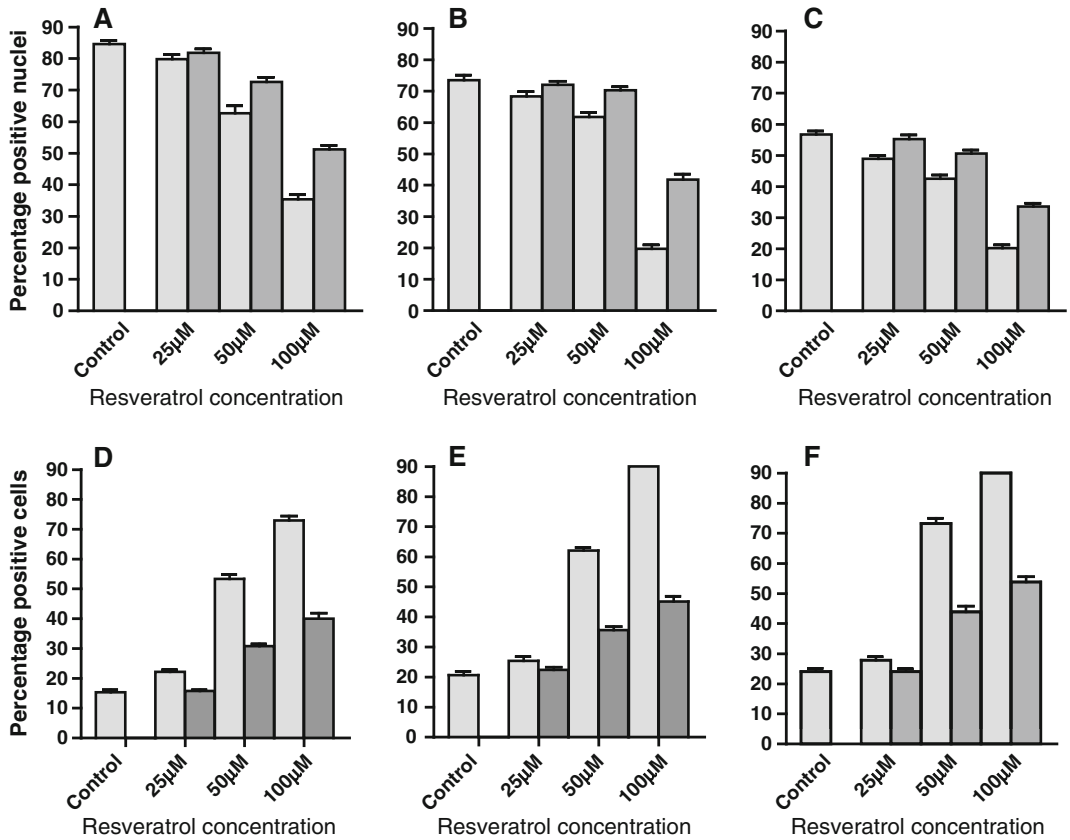

Fig. 5 Pretreatment with SB203580 is protective against resveratrol-induced senescence. Replicate (n=3) cultures of MRC5 fibroblasts at 30 (**a** and **d**), 43 (**b** and **e**) and 56 (**c** and **f**) population doublings were exposed to 25, 50 or 100 μM resveratrol for 48 h either alone (*light grey bars*) or following pre-treatment with 10 μM SB203580 for 30 min (*dark grey bars*). Protection was assayed by staining for both Ki67 (**a**–**c**) and SA-β-galactosidase (**d**–**f**). Significant protection was observed in both assays for resveratrol concentrations of 50 μM and above (p<0.05, data presented ±SEM)

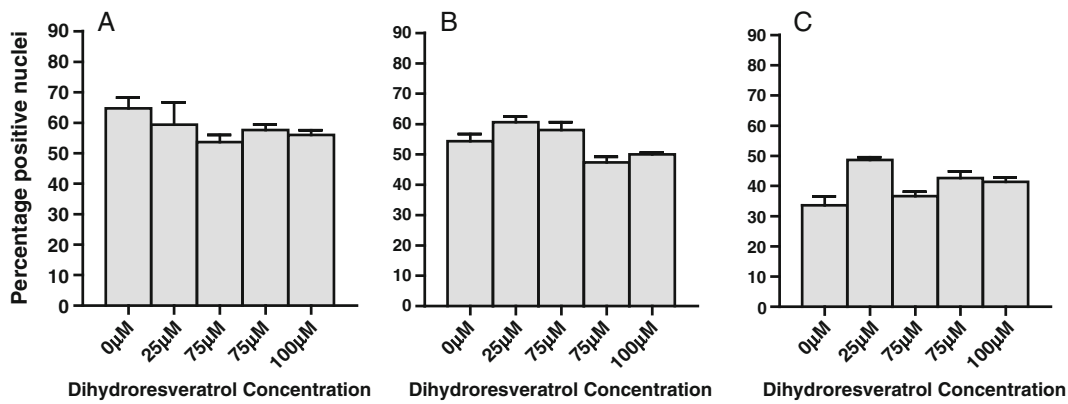

Fig. 6 Effect of dihydroresveratrol on the growth fraction of MRC5 fibroblast cultures. Replicate cultures (n=3) at **a** 46 population doublings, **b** 49 population doublings and **c** 56 population doublings were exposed to a vehicle only control or 25, 50 or 100 μM dihydroresveratrol for 48 h. The y-axes show the percentage of Ki67 positive nuclei at each concentration and population doubling level (±SEM). No significant reduction in growth fraction compared to control was seen at any concentration or population doubling level assayed

by a short pretreatment with the semi-selective p38MAP kinase inhibitor SB203580. This is an important finding because whilst p38-dependent arrest has been shown to occur in some cancer cell lines it also has been shown in others that resveratrol-induced growth arrest occurs in a p38-independent manner through the generation of endogenous ceramide, a mechanism which also operates in normal cells (Ulrich et al. 2007, Venable et al. 1995, Venable and Yin 2009). Our data are therefore consistent with a model (see Fig. 7) in which the major response to exposure to resveratrol in normal fibroblasts is a rapid, p38-dependent growth arrest similar to that seen in fibroblasts taken from patients with the accelerated ageing disease Werner's syndrome or treated with hydrogen peroxide (Davis et al. 2005, Zdanov et al. 2006).

Rendering cells incapable of division might, under certain circumstances, be beneficial (e.g. by preventing restenosis) provided that the phenotype of the arrested cells remains benign. Unfortunately, entry into senescence in fibroblasts is frequently associated with a shift to a highly proinflammatory phenotype which has the potential to compromise the function of any tissue in which such cells accumulate (Bird et al. 2003, Herbig et al. 2006, Kipling et al. 2009, Burton 2009). In a reconstituted human skin model, the introduction of senescent fibroblasts has been shown to induce changes reminiscent of those seen in skin from the elderly (Funk et al. 2000). Based on resveratrol's ability to activate SirT1, Cao et al. (2009) proposed that it, and related compounds, might be useful topical agents for the prevention of skin ageing. However, in the light of our data and those of Funk et al. (2000), we consider it more likely that the topical application of resveratrol at concentrations above 25 μM will produce deleterious changes in skin structure. We caution against any such use until more extensive tests have been performed.

An uncritical reading of our data might seem to suggest that dietary supplementation with resveratrol could trigger adverse effects through the induction of cellular senescence in a range of tissues. We consider this highly unlikely because although it is absorbed well, resveratrol taken orally is rapidly metabolised and is thus not present at detectable concentrations in plasma (Walle et al. 2004). We tested the effects of resveratrol at very low doses and found no general evidence that resveratrol has any negative effect on the growth fraction of human fibroblasts just as we had previously observed with vascular smooth muscle cells (Burton et al. 2007). Thus, we do not consider it likely that induction of cellular senescence in vivo could occur through this route.

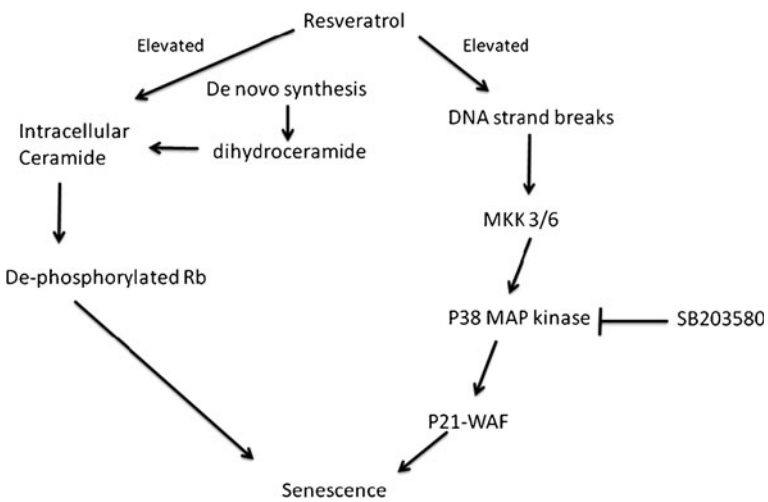

Fig. 7 Simple schematic for the action of resveratrol at high doses. Resveratrol at high doses can produce growth arrest either through the elevation of intracellular ceramide and its effects on the phosphorylation status of retinoblastoma or through the activation of p38MAP kinase probably through the generation of DNA strand breaks leading to growth arrest *via* elevated levels of the cyclin dependent kinase inhibitor p21WAF. (Diagram summarises our own data together with that of Ulrich et al. (2007), Venable et al. (1995), Davis et al. (2005) and Delgado et al. (2006))

This being said, a decline in the growth fraction was noted in one cell strain (Ek1.Br) treated at a dose of 5 μM resveratrol (though not at 10 μM). Interestingly, enhanced proliferation was observed for MRC5 fibroblasts treated with 10 μM resveratrol compared to 5 μM resveratrol. In this respect, our results are in contrast with those of Giovannelli et al. (2010) who report a 'tendency' for the growth of MRC5 fibroblasts to be inhibited at this dose. No data was shown to support this but the effect was clearly sufficiently large and reproducible for the researchers to select 5 μM resveratrol as the concentration at which to conduct their subsequent studies. It would appear that at low doses, the balance between the pro-proliferative and growth suppressive effects of resveratrol may be very fine and that care must be taken when comparing low dose studies using different cell strains.

Resveratrol metabolites are detectable at relatively high concentrations in human plasma (~2 μM, following a single 25 mg oral dose) and at least one of them (dihydroresveratrol) has been proposed to display antiproliferative effects of similar potency to resveratrol itself in SCC-9 cells (Walle et al. 2004). However, we synthesised pure dihydroresveratrol and found that this metabolite has no effect on the growth fraction of MRC5 fibroblasts at concentrations of up to 100 μM. Equally, the sulphated and glucuronidated metabolites of resveratrol have short residence times within cells (Henry et al. 2005; Lançon et al. 2007), and thus seem unlikely candidates for the induction of senescence, although it is possible that they have distinct effects on population dynamics.

In conclusion, our data are not consistent with postulated models of resveratrol action that invoke alterations in cell turnover as the primary mechanism by which it mediates "anti-ageing" effects. Resveratrol concentrations in vivo simply seem to be too low for the molecule to either stimulate or inhibit proliferation, and we have shown no alteration in apoptosis rates in cells exposed to micromolar concentrations of the molecule in vitro (Burton et al. 2007 and unpublished observations). Ford et al. have recently proposed that alterations in DNA methylation in response to very low concentrations of resveratrol may mediate some of its in vivo effects (Wakeling et al. 2009). This seems to us to be a plausible mechanism of action for this class of molecules and it is consistent with very recent work from Giovannelli et al. (2010) who found that chronic treatment of MRC5 fibroblasts with 5 μm resveratrol reduced senescence associated beta galactosidase staining levels without significantly increasing the maximum population doubling levels attained by the cultures. We have recently published microarray datasets for the HCA2 and Ek1.Br fibroblasts strains used in this study (Kipling et al. 2009) and further transcriptomic analysis following treatment with physiologically reflective levels of resveratrol and dihydroresveratrol would allow Ford's hypothesis to be tested.

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References

Bass TM, Weinkove D, Houthoofd K, Gems D, Partridge L (2007) Effects of resveratrol on lifespan in *Drosophila melanogaster* and *Caenorhabditis elegans*. Mech Age Dev 128:546–552

Barger JL, Kayo T, Vann JM, Arias EB, Wang J, Hacker TA, Wang Y, Raederstorff D, Morrow JD, Leeuwenburgh C, Allison DB, Saupe KW, Cartee GD, Weindruch R, Prolla TA (2008) A low dose of dietary resveratrol partially mimics caloric restriction and retards aging parameters in mice. PLoS ONE 3:e2264

Bellance N, Benard G, Furt F, Begueret H, Smolková K, Passerieux E, Delage JP, Baste JM, Moreau P, Rossignol R (2009) Bioenergetics of lung tumors: alteration of mitochondrial biogenesis and respiratory capacity. Int J Biochem Cell Biol 41:2566–2577

Bird J, Ostler EL, Faragher RGA (2003) Can we say that senescent cells cause aging? Exp Gerontol 38(11–12):1319–1326

Burton DGA, Sheerin A, Ostler EL, Smith K, Giles PJ, Lowe J, Rhys-Williams W, Kipling DG, Faragher RGA (2007) Cyclin D1 over-expression permits the reproducible detection of senescent human vascular smooth muscle cells. Ann NY Acad Sci 1119:20–31

Burton DGA (2009) Cellular senescence, ageing and disease. AGE 31(1):1–9

Cao C, Lu S, Kivlin R, Wallin B, Card E, Bagdasarian A, Tamakloe T, Wang WJ, Song X, Chu WM, Kouttab N, Xu A, Wan Y (2009) SIRT1 confers protection against UVB- and H2O2-induced cell death via modulation of p53 and JNK in cultured skin keratinocytes. J Cell Mol Med 13 (9b):3632–3643

Davis T, Baird DM, Haughton MF, Jones CJ, Kipling D (2005) Prevention of accelerated cell aging in Werner syndrome using a p38 mitogen-activated protein kinase inhibitor. J Gerontol Biol Sci Med Sci 60:1386–1393

Delgado A, Casas J, Llebaria A, Abad JL, Fabrias G (2006) Inhibitors of sphingolipid metabolism enzymes. Biochim Biophys Acta 1758:1957–1977

Ferry-Dumazet H, Garnier O, Mamani-Matsuda M, Vercauteren J, Belloc F, Billiard C, Dupouy M, Thiolat D, Kolb JP, Marit G, Reiffers J, Mossalayi MD. (2002) Resveratrol inhibits the growth and induces the apoptosis of both normal and leukemic hematopoietic cells. Carcinogenesis 23:1327-33

Funk WD, Wang CK, Shelton DN, Harley CB, Pagon GD, Hoeffler WK (2000) Telomerase expression restores dermal integrity to in vitro-aged fibroblasts in a reconstituted skin model. Exp Cell Res 258:270–278

Giovannelli L, Pitozzi V, Jacomelli M, Mulinacci N, Laurenzana A, Dolara P, Mocali A (2010) Protective effects of resveratrol against senescence-associated changes in cultured human fibroblasts. J Gerontol A Biol Sci Med Sci (in press)

Grove GL, Cristofalo VJ (1977) Characterization of the cell cycle of cultured human diploid cells: effects of aging and hydrocortisone. J Cell Physiol 90:415–422

Henry C, Vitrac X, Decendit A, Ennamany R, Krisa S, Merillon JM (2005) Cellular uptake and efflux of *trans*-piceid and its aglycone *trans*-resveratrol on the apical membrane of human intestinal Caco-2 cells. J Agric Food Chem 53:798–803

Herbig U, Ferreira M, Condel L, Carey D, Sedivy JM (2006) Cellular senescence in aging primates. Science 311:1257

Howitz KT, Bitterman KJ, Cohen HY, Lamming DW, Lavu S, Wood JG, Zipkin RE, Chung P, Kisielewski A, Zhang LL, Scherer B, Sinclair DA (2003) Small molecule activators of sirtuins extend *Saccharomyces cerevisiae* lifespan. Nature 425:191–196

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

Kipling D, Jones DL, Smith SK, Giles PJ, Jennert-Burston K, Ibrahim B, Sheerin AN, Evans AJ, Rhys-Willams W, Faragher RG (2009) A transcriptomic analysis of the EK1. Br strain of human fibroblastoid keratocytes: the effects of growth, quiescence and senescence. Exp Eye Res 88 (2):277–285

Lançon A, Hanet N, Jannin B, Delmas D, Heydel J-M, Lizard G, Chagnon M-C, Artur Y, Latruffe N (2007) Resveratrol in human hepatoma HepG2 Cells: metabolism and inducibility of detoxifying enzymes. Drug Metab Dispos 35:699–703

Pascual-Martí MC, Salvador A, Chafer A, Berna A (2001) Supercritical fluid extraction of resveratrol from grape skin of *Vitis vinifera* and determination by HPLC. Talanta 54:735–740

Pearson KJ, Baur JA, Lewis KN, Peshkin L, Price NL, Labinskyy N, Swindell WR, Kamara D, Minor RK, Perez E, Jamieson HA, Zhang Y, Dunn SR, Sharma K, Pleshko N, Woollett LA, Csiszar A, Ikeno Y, Le Couteur D, Elliott PJ, Becker KG, Navas P, Ingram DK, Wolf NS, Ungvari Z, Sinclair DA, de Cabo R (2008) Resveratrol delays age-related deterioration and mimics transcriptional aspects of dietary restriction without extending life span. Cell Metab 8(2):157–168

Rusin M, Zajkowicz A, Butkiewicz D (2009) Resveratrol induces senescence-like growth inhibition of U-2 OS cells associated with the instability of telomeric DNA and upregulation of BRCA1. Mech Ageing Dev 30(8):528–537

Saiko P, Szakmary A, Jaeger W, Szekeres T. (2008) Resveratrol and its analogs: defense against cancer, coronary disease and neurodegenerative maladies or just a fad? Mutat Res. 658:68-94

Stivala LA, Savio M, Carafoli F, Perucca P, Bianchi L, Maga G, Forti L, Pagnon UM, Albini A, Prosper E (2001) Specific structural determinants are responsible for the antioxidant activity and the cell cycle of resveratrol. J Biol Chem 276:22586–22594

Ulrich S, Huwiler A, Loitsch S, Schmidt H, Stein JM (2007) De novo ceramide biosynthesis is associated with resveratrol-induced inhibition of ornithine decarboxylase activity. Biochem Pharmacol 74:281–289

Valenzano DR, Terzibasi E, Genade T, Cattaneo A, Domenici L, Cellerino A. (2006) Resveratrol prolongs lifespan and retards the onset of age-related markers in a short-lived vertebrate. Curr Biol. 16(3):296-300

Venable ME, Lee JY, Smyth MJ, Bielawska A, Obeid LM (1995) Role of ceramide in cellular senescence. J Biol Chem 270:30701–30708

Venable ME, Yin X (2009) Ceramide induces endothelial cell senescence. Cell Biochem Funct 27:547–551

Wakeling LA, Ions LJ, Ford D (2009) Could Sirt1-mediated epigenetic effects contribute to the longevity response to dietary restriction and be mimicked by other dietary interventions? Age (Dordr) (in press)

Walle T, Hsieh F, DeLegge MH, Oatis JE Jr, Walle UK (2004) High absorption but very low bioavailability of oral resveratrol in humans. Drug Metab Dispos 32:1377–1382

Wood JG, Rogina B, Lavu S, Howitz K, Helfand SL, Tatar M, Sinclair D (2004) Sirtuin activators mimic caloric restriction and delay ageing in metazoans. Nature 430:686–689

Zdanov S, Debacq-Chainiaux F, Remacle J, Toussaint O (2006) Identification of p38MAPK-dependent genes with changed transcript abundance in H_2O_2-induced premature senescence of IMR-90 hTERT human fibroblasts. FEBS Lett 580:6455–6463