Research Article

Resveratrol-fortification of red wine does not provide greater inhibition of human lung cancer cell survival compared to non-fortified wine

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Abstract

Lung cancer is the leading cause of cancer-related deaths, and individuals with this disease often develop resistance to conventional cytotoxic therapies. Red wine and its polyphenolic component resveratrol, have been shown to have anticancer effects. Wines fortified with resveratrol have been marketed as having additional health benefits because of their increased polyphenolic content, however no studies exist examining this claim. The aim of the present study was to explore the effects of resveratrol-fortified red wine on lung cancer cell survival. Human NSCLC A549 cells were treated with varying concentrations of red wine with or without *trans*-resveratrol fortification. Cell survival was assessed using clonogenic assays and immunoblotting was used to explore the effects on Akt and ERK signaling molecules. Red wine significantly inhibited cell survival at concentrations as low as 0.02%, and significantly reduced phosphorylation of both Akt and ERK. No significant differences were seen between regular and resveratrol-fortified red wine. These data suggest that red wine may have considerable cancer preventive potential, however it does not support the use of resveratrol-fortified wine for additional health benefits.

Introduction

As a single entity, cancer is the largest cause of mortality globally. Lung cancer accounts for the most cancer related deaths (Stewart & Wild 2014) with non-small cell lung cancer (NSCLC) accounting for the majority (85%) of lung cancer cases (Jones *et al.* 2015). Unfortunately, the outcome of NSCLC is very poor with fewer than 20% of patients reaching a 5 year survival and this statistic exists despite the use of aggressive chemotherapy and/or radiation treatment (Jones *et al.* 2015). NSCLC often develops resistance to conventional cancer treatments (Rothschild 2015, Thomas *et al.* 2015), strongly indicating an urgent need to identify new effective strategies for its prevention and treatment.

Cancer in general is characterized by enhanced growth factor signaling leading to increased proliferation and reduced programmed cell death (apoptosis) (Hanahan & Weinberg 2011). The phosphatidylinositol 3-kinase (PI3K)-Akt signaling cascade is activated downstream of growth factor receptors, following their stimulation upon ligand binding, leading to cell survival (inhibition of apoptosis) and enhanced proliferation (Manning & Cantley 2007). Indeed when Akt is activated apoptosis is inhibited due to phosphorylation and degradation of the pro-apoptotic protein Bad (Manning & Cantley 2007) and phosphorylation of murine double minute 2 (MDM2) homologue, which then translocates into the nucleus and inhibits p53 levels (Mayo & Donner 2001). On the other hand, activated Akt leads to downstream activation of the mammalian target of rapamycin (mTOR) and p70 S6 kinase, resulting in stimulation of protein synthesis, growth and proliferation (Manning & Cantley 2007).

Many studies have shown that Akt levels and its activation are increased in many cancer cells and human tumors (Brognard *et al.* 2001, Liu *et al.*2009), and when Akt signaling is inhibited cancer cell proliferation is reduced and apoptosis is increased (Brognard *et al.* 2001, Ermoian *et al.* 2002, Pérez-Tenorio & Stål 2002). Akt is a proto-oncogene and its activation is also implicated in the induction of resistance to chemo - and radio-therapy (Brognard *et al.* 2001, Liu *et al.*

Table 1. Basic physicochemical composition and phenolic content of Cabernet Sauvignon wines. Data represent mean values of duplicate measurements from duplicate bottles ± standard deviations. Means sharing the same letter do not differ significantly (Fisher's Protected LSD $_{0.05}$). Modified from Gaudette & Pickering (2011).

	trans- RSV (mg/L)	*cis*-RSV (mg/L)	Total Phenolics (A_{280} nm^{-4})	Free SO_2 (mg/L)	Total Red Pigments (A_{520} nm/$AHCl_{520}$ nm x 100)	Titratable Acidity (g/L)	pH	Antioxidant Capacity (Trolox™ equivalent)
– RSV	< 0.49	< 0.13	22.2 a ± 0.38	8.40 a ± 1.53	10.8 a ± 0.13	6.06 a ± 0.20	3.09 a ± 0.02	30.33 a ± 0.14
+20 mg/L RSV	12.9 a ± 0.16	< 0.13	24.7 b ± 1.47	6.40 b ± 0.00	11.6 b ± 0.59	5.98 a ± 0.17	3.06 a ± 0.00	32.9 ab ± 1.36
+200 mg/L RSV	146.0 b ± 1.88	0.33 ± 0.06	33.6 c ± 0.65	6.80 b ± 0.80	11.1 c ± 0.15	5.49 b ± 0.46	3.08 a ± 0.01	35.2 b ± 0.34

2007). Based on this evidence, the scientific community has focused on finding chemicals that will target/ inhibit Akt as a strategy to prevent and treat cancer (Ciuffreda *et al.* 2014).

The Ras- mitogen-activated protein kinase (MAPK) signaling cascade is also activated downstream of growth factor receptor stimulation, leading to increased proliferation. The extracellular signal-regulated kinase (ERK), a member of the MAPK family, is activated in cancer leading to enhanced cell proliferation and survival (Ciuffreda *et al.* 2014, Roberts & Der 2007). Mutations to the Ras and Raf oncogenes, which are located upstream in this signaling cascade and thus control activation of ERK, are frequent in human cancers and notably in lung cancers (Davies *et al.*, 2002), highlighting the importance of targeting this pathway using novel anticancer agents (Gentry *et al.* 2013).

Compounds of plant origin and food components have attracted scientific attention for use as agents for cancer prevention and treatment. Recent studies indicate that wine may have anticancer properties (Barron *et al.* 2014, Elattar & Virji 1998, Wallenborg *et al.* 2009). Red wine inhibited the proliferation of human prostate cancer cells (Kampa *et al.* 2000) and human oral squamous carcinoma (SCC-25) cells (Elattar & Virji 1998) while it increased lung, colon and cervical cancer cell death (Wallenborg *et al.* 2009). In a recent study by our group we found a significant inhibition of human lung cancer cell proliferation and survival by wine (Barron *et al.* 2014).

The polyphenol resveratrol is found at a relatively high concentration in red wine (1-5 mg/L) and has been proposed to be responsible for the reduced incidence of cardiovascular disease associated with moderate wine consumption (German & Walzem 2000, Zordoky *et al.* 2015). Similarly, it is thought that the potential anticancer properties of wine may be attributable to its resveratrol content, and both *in vitro* and *in vivo* studies with resveratrol support such thinking (Aluyen *et al.* 2012, Jang *et al.* 1997, Yang *et al.* 2014). Resveratrol has been shown to inhibit growth of multiple myeloma (Sun *et al.* 2006), uterine (Sexton *et al.* 2006), pancreatic (Golkar *et al.* 2007) and lung (Kubota *et al.* 2002) cancer cells. In addition, in animals *in vivo*, resveratrol inhibited diethylnitrosamine (DENA)-induced liver cancer (Bishayee & Dhir 2009), colorectal cancer (Tessitore *et al.* 2000) and enhanced radio-sensitivity of prostate cancer (Rashid *et al.* 2011). Resveratrol is extracted from grape skins into red wine during vinification, where it is found in four forms: *trans*- and *cis*-aglycone, and *trans*- and *cis*-glycoside. The relative concentrations of these forms are mediated by isomerisation and hydrolytic reactions, although the *trans*-form of the aglycone is more abundant than the *cis* (Mattivi *et al.* 1995).

The evidence of the anticancer properties of resveratrol has prompted the production of resveratrol-fortified wines, which are being marketed as having enhanced health benefits (Norrie 2009), and is consistent with the recent increase in functional foods fortified with phenolic compounds (Gaudette & Pickering 2013). However the biological effects and whether an added benefit is provided by such wine is not known, and there are no studies examining the effects of resveratrol-fortified wine on cancer. In the present study we examined the effects of red wine with or without added *trans*-resveratrol (at two concentrations) on lung cancer cell survival and on the Akt and ERK signaling cascades.

Materials and Methods

Materials

Human A549 NSCLC cells and MRC5 normal lung fibroblasts were purchased from American Type Cul-

ture Collection (ATCC). Cell culture (RPMI & DMEM) media, fetal bovine serum (FBS), trypsin, and antibiotic were purchased from GIBCO (Burlington, ON, Canada). Crystal violet dye and 10% formalin were purchased form Sigma Aldrich (Oakville, ON, Canada). Total and phospho-specific Akt and ERK antibodies and secondary antibodies were purchased from New England Biolabs (Mississauga, ON, Canada). Primary β-actin antibody was purchased from Santa Cruz Biotechnology (Dallas, TX, U.S.A). Red wine (Cabernet Sauvignon) with or without added resveratrol was prepared at the Cool Climate Oenology and Viticulture Institute (CCOVI), Brock University. *trans*-Resveratrol (99% pure food grade; Chromadex, Irvine, California, USA) was added to wine at 20mg/L or 200 mg/L prior to bottling as previously described (Gaudette & Pickering, 2011). The basic physico-chemical composition and phenolic content of the wines were measured as previously described (Gaudette & Pickering 2011).

Cell Culture and Treatment

A549 cells were grown in RPMI media and MRC5 cells were grown in DMEM media, each supplemented with 10% (v/v) FBS and 1% (v/v) antibiotic-antimycotic solution (100 U/ml penicillin, 100 µg/ml streptomycin, and 250 ng/ml amphotericin B) in a humidified atmosphere of 5% CO_2- 95% air at 37°C. Cells were seeded in 6 well plates and treated with the indicated concentrations of red wine with or without added resveratrol for the indicated times as stated in each figure.

Clonogenic Assay

Clonogenic assays were performed as described previously (Barron *et al.* 2014). Cells (800-1000) were counted using a haemocytometer, seeded in triplicate and allowed to adhere overnight. The next day cells were treated with the indicated concentrations of red wine with or without added resveratrol (mixed in 10% FBS containing media), followed by incubation for 7 days. On day 7 cells were fixed and stained with 0.05% (w/v) methylene blue and colonies (> 50 cells) were counted. Results are expressed as the surviving fraction compared to untreated control.

Crystal Violet Assay

Cells (800-1000) were counted using a haemocytometer, seeded in triplicate in 96 well plates and allowed to adhere overnight. The next day cells were treated with the indicated concentrations of red wine with or without added resveratrol (mixed in 10% FBS containing media) and incubated for 72 hours. Following treatment cells were fixed with 10% formalin, stained with crystal violet, rinsed with tap water and allowed to dry overnight. The next day, adhered crystal violet stain was solubilized and absorbance was measured at 570nm using the KC4 plate reader. Results are expressed as percent of untreated control.

Immunoblotting

A549 or MRC5 cells were seeded in 6-well plates at a high density and allowed to adhere overnight. The following day the media in the plates was replaced with 0% FBS containing media and cells were serum deprived for 24h. Cells were then treated with the indi-

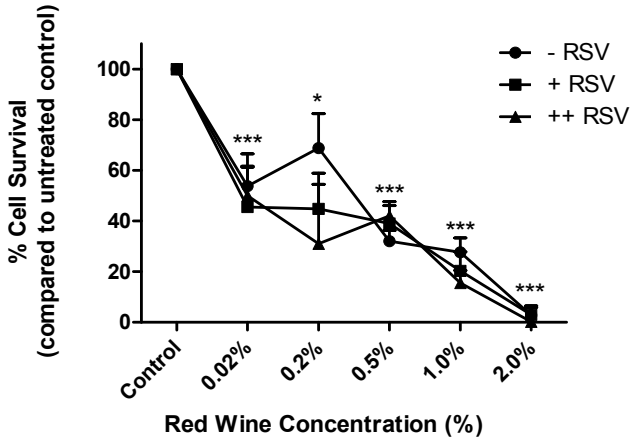

Figure 1. Red wine inhibits cell survival in A549 NSCLC cells. Cells were incubated without or with the indicated concentrations of Cabernet Sauvignon wine for 7 days followed by fixing and staining with 0.05% methylene blue. Colonies containing more than 50 cells were counted. Results are expressed as the surviving fraction ± SEM compared to untreated control of 3-4 independent experiments. *p<0.05, ***p<0.001 vs. control

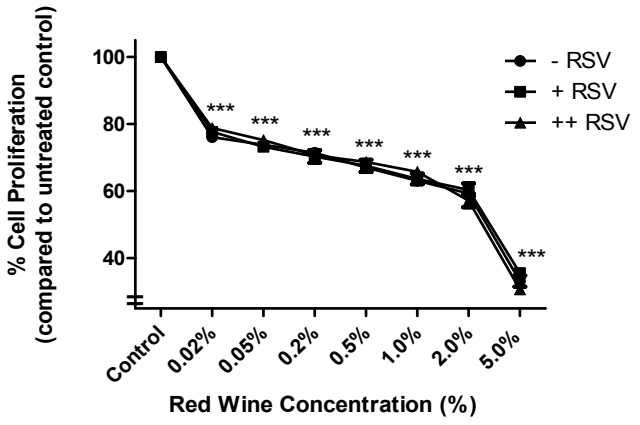

Figure 2. Red wine inhibits cell proliferation in A549 NSCLC cells. Cells were incubated without or with the indicated concentrations of Cabernet Sauvignon wine for 72 hours, fixed and stained with crystal violet dye. Adhered dye was solubilized and absorbance was read at 570nm. Results are expressed as the % cell proliferation ± SEM compared to untreated control of 3 independent experiments. ***p<0.001 vs. control

Table 2. Calculated final *trans*-resveratrol concentration in the media containing different wine concentrations (%)

Media [Wine]%:	0.02	0.2	0.5	1	2
-RSV (2.14μM)	0.429nM	4.29nM	10.73nM	21.46nM	42.92nM
+RSV (56.5μM)	11.3nM	113nM	282.5nM	565nM	1.13μM
++RSV (639.68μM)	128nM	1.28μM	3.20μM	6.40μM	12.79μM

cated concentrations of red wine with or without added resveratrol for another 24h (in 0% FBS containing media). After treatment, cell lysates were prepared followed by separation of 20 μg of protein sample by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein was then transferred to polyvinylidene fluoride (PVDF) membrane and blocked with 5% milk. Membranes were incubated with total- or phospho- specific Akt, ERK or β-actin antibodies overnight. The next day, secondary antibody was applied to the membrane for 1h and bands were detected using Biorad Clarity ECL substrate. Densitometric analysis was performed using Image J software. Results are expressed in arbitrary densitometric units as the mean ± SEM of control.

Statistical Analysis

The results are the mean ± SEM of the indicated number of independent experiments. Analysis of variance (ANOVA) with Tukey post-hoc testing was used. Statistical significance was assumed at $p<0.05$. GraphPad Prism 6 Software was used for statistical analysis.

Results

The physicochemical composition of the red wines used is shown in Table 1. We aimed to have low and high levels of resveratrol fortification, thus 20mg/L or 200 mg/L of resveratrol was added, with the latter value reflecting the approximate upper limit of solubility. As reported by Gaudette & Pickering (2011), a small initial decrease in content occurred after bottling -possibly due to the known propensity of polyphenols to self-associate and complex with other wine constituents - yielding final and stable concentrations of 12.9 mg/L and 146 mg/L, respectively. For the rest of the paper unfortified wine is also referred to as – RSV, while wine with low and high levels of resveratrol fortification are referred to as + RSV and ++ RSV respectively.

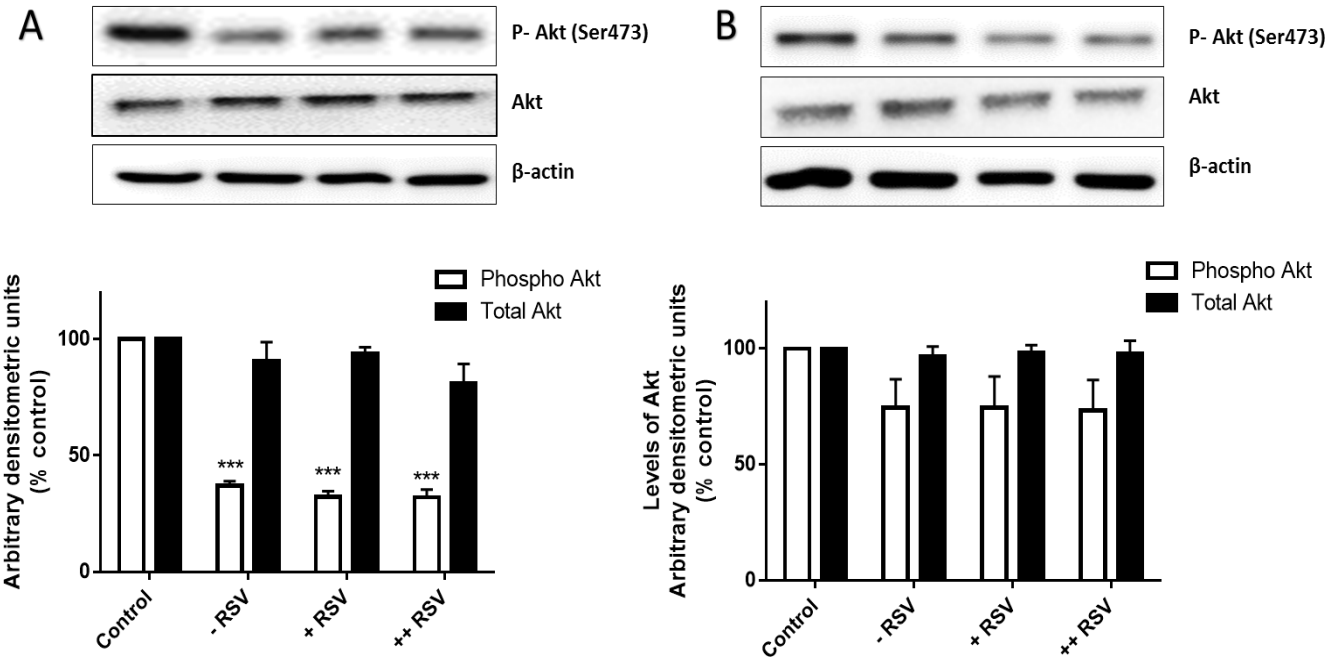

Figure 3. Effect of red wine on Akt signaling in A549 NSCLC cells. Whole cell lysates were prepared from A549 cells that were serum-deprived for 24h then treated without or with A) 1% or B) 0.02% wine for 24h. Cell lysates (20μg) were resolved by SDS-PAGE and immunoblotted with total- or phospho- specific antibodies against Akt. A representative immunoblot is shown. The densitometry of the bands expressed in arbitrary units was measured using Image J software. Protein levels are expressed as a percentage of the control. Results represent mean ± SEM of 3 independent experiments. ***$p<0.001$ vs. control

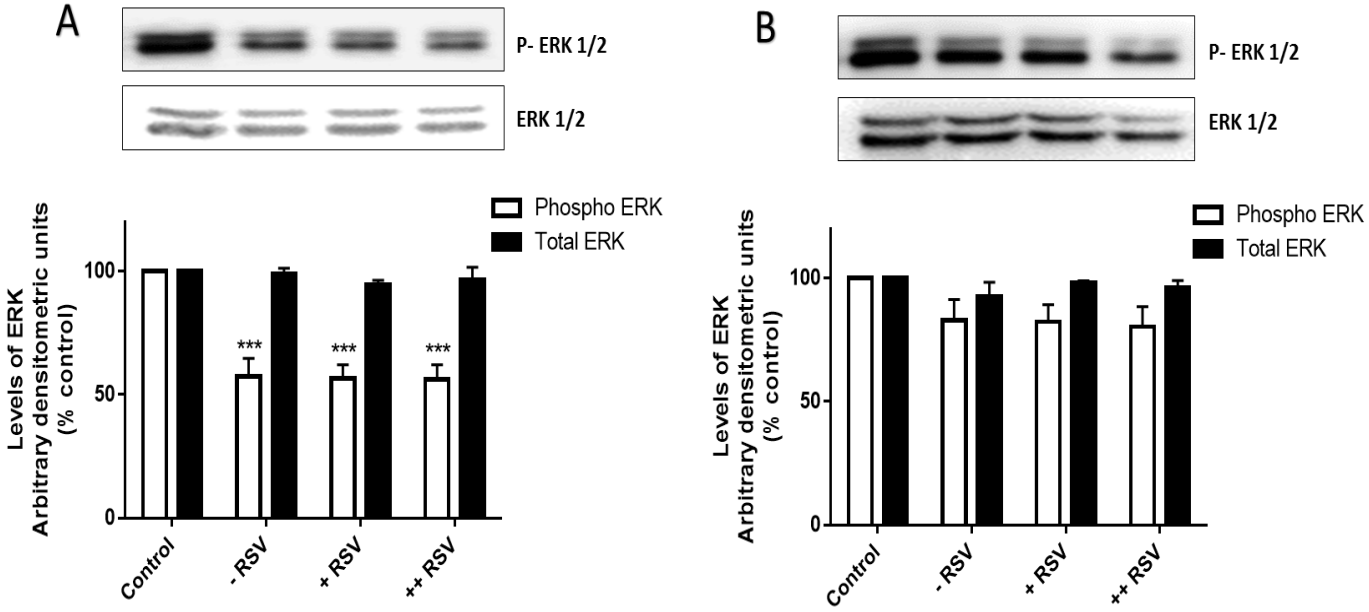

Figure 4. Effect of red wine on ERK signaling in A549 NSCLC cells. Whole cell lysates were prepared from A549 cells that were serum-deprived for 24h then treated without or with A) 1% or B) 0.02% wine for 24h. Cell lysates (20μg) were resolved by SDS-PAGE and immunoblotted with total- or phospho- specific antibodies against ERK. A representative immunoblot is shown. The densitometry of the bands expressed in arbitrary units was measured using Image J software. Protein levels are expressed as a percentage of the control. Results represent mean ± SEM of 3 independent experiments. ***p<0.001 vs. control

Effects of resveratrol-fortified wine on lung cancer cell survival and proliferation

A549 lung cancer cells were exposed to media without or with different concentrations (0.02, 0.2, 0.5, 1, 2%) of red wine, and clonogenic cell survival was measured. Exposure to red wine resulted in a dose-dependent inhibition of clonogenic cell survival. A significant inhibition was seen at 0.02% red wine (-RSV: 53.8±3.8% of untreated control) (Figure 1). Interestingly, the same level of inhibition of clonogenic survival was seen with wine fortified with resveratrol (+RSV: 45.5±8.0%; ++RSV: 50±9.5% of untreated control) (Figure 1). Red wine at 2% abolished most of the ability of cells to create surviving colonies, and again no statistical differences were seen between fortified (+RSV: 3.0±1.7%; ++RSV: 0.3±0.3% of untreated control) and unfortified wine (-RSV: 2.8±1.6% of untreated control) (Figure 1). These data clearly indicate that red wine induces a significant inhibition of lung cancer cell survival and this effect is not potentiated by the addition of resveratrol into the wine.

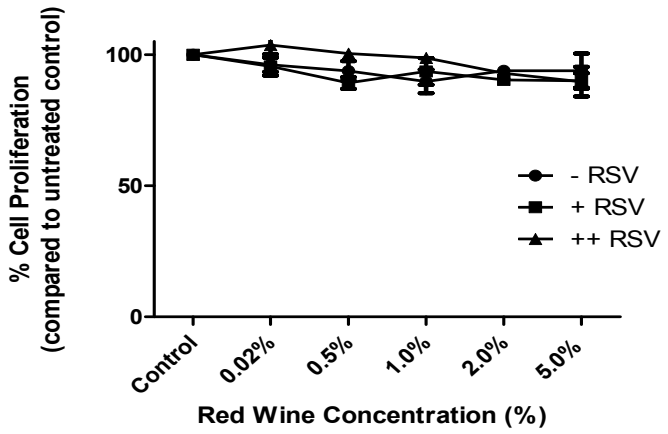

Figure 5. Red wine does not inhibit cell proliferation in non-cancerous MRC5 lung cells. MRC5 normal lung fibroblasts were incubated without or with the indicated concentrations of Cabernet Sauvignon wine for 72 hours, fixed and stained with crystal violet dye. Adhered dye was solubilized and absorbance was read at 570nm. Results are expressed as the % cell proliferation ± SEM compared to untreated control of 3 independent experiments.

To examine the effect of red wine on A549 lung cancer cell proliferation, the crystal violet assay was used. Cells were exposed to media without or with different concentrations of wine (0.02, 0.05, 0.2, 0.5, 1, 2%) and a dose-dependent inhibition of cell proliferation was seen (Figure 2). Red wine induced a significant inhibition at 0.02% (-RSV: 76.1±1.5%, +RSV: 77.7±1.2%; ++RSV: 78.8±1.2% of untreated control) without any differences observed with resveratrol fortification. At 5% fortified and unfortified red wine inhibited cell proliferation to the same extent (-RSV: 33.2±1.2%; +RSV: 35.6±0.7%; ++RSV: 30.7±1.1% of untreated control). The IC50 of unfortified red wine was 2.95% and was not different than fortified wine.

Based on the concentration of resveratrol in the wines used and the molecular weight of resveratrol

(228.24 g/mol), we calculated the final concentration of resveratrol in the media containing different wine concentrations (Table 2). These values are the calculated final concentrations of resveratrol the cells were exposed to. Although, as it can be seen from Table 2, the cells were exposed to significantly different resveratrol concentrations, the level of inhibition of clonogenic survival was not significantly different (Figure 1). For example, cells that were treated with 0.02% fortified red wine were exposed to 11.3 nM or128 nM resveratrol but the inhibition of clonogenic survival was 45.5±8.1 and 50.0±9.5% of control respectively, and not different than the inhibition seen with unfortified wine exposed to only 0.429 nM resveratrol (-RSV: 53.8±3.8% of untreated control). The same was observed for the inhibition of proliferation.

Effects of resveratrol-fortified wine on Akt phosphorylation/activation.

As reported by our group previously (Barron *et al.* 2014), control/untreated A549 cells have high levels of Akt phosphorylation which correlate with the activation of this enzyme (Manning & Cantley 2007). Treatment of the cells with 1% red wine significantly inhibited Akt phosphorylation (-RSV: 36.9±2.0% of control) (Figure 3A) and low or high resveratrol fortification of red wine did not result in any further reduction in Akt phosphorylation (+RSV: 32.2±2.4%; ++RSV: 32.1±3.3% of control). Treatment of the cells with a much lower concentration (0.02%) of wine showed a tendency to decrease Akt phosphorylation, but it was not statistically different than control even in the groups treated with the fortified wine (-RSV: 74.6±12.3%; +RSV: 74.4±13.7%; ++RSV: 73.4±13.2% of control) (Figure 3B). Total Akt levels were not significantly changed by any treatment (Figure 3A, 3B).

Effects of resveratrol-fortified wine on ERK phosphorylation/activation.

Untreated A549 cells showed high levels of basal ERK phosphorylation, and red wine (1%) with or without resveratrol fortification reduced basal ERK phosphorylation significantly (-RSV: 57.4±7.4%; +RSV: 56.6±5.6%; ++RSV: 56.2±6.0% of control) without affecting the total levels of this enzyme (Figure 4A). Similarly to Akt data, treatment of the cells with red wine fortified with resveratrol did not result in a greater response compared to the response seen with wine without resveratrol fortification. Treatment of the cells with a lower concentration of wine (0.02%) did not result in significant inhibition of ERK phosphorylation even in the groups treated with fortified wine (-RSV: 82.9±8.9%; +RSV: 82.5±6.8%; ++RSV: 80.4±8.1% of control) (Figure 4B). Total ERK levels were not significantly changed by any treatment (Figure 4A, 4B).

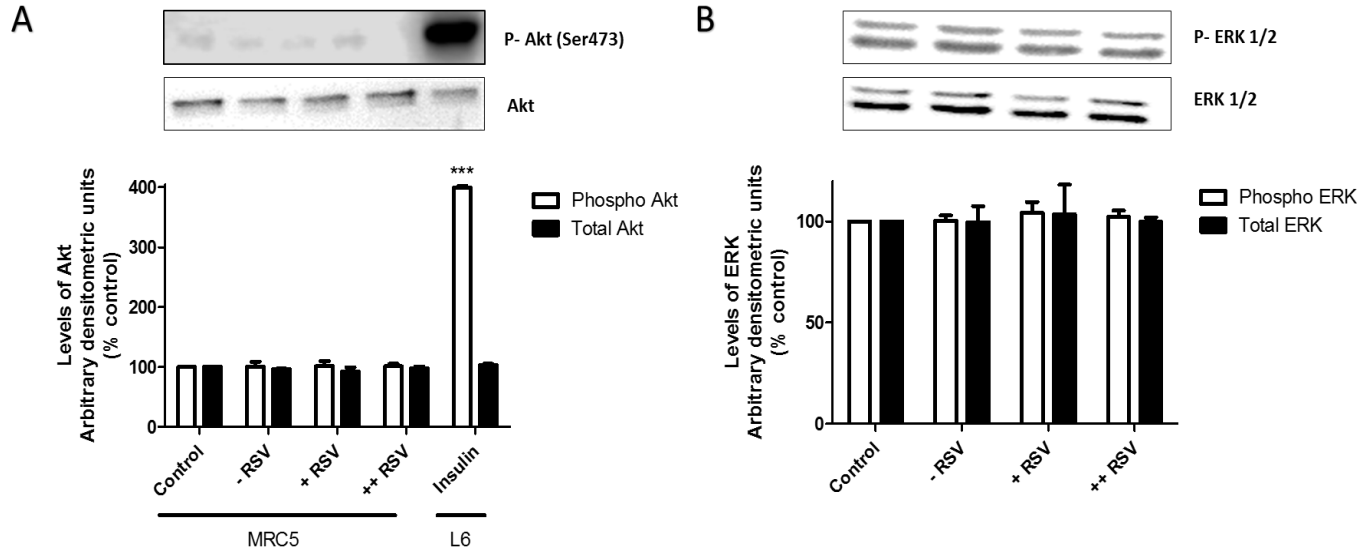

Figure 6. Effect of red wine on Akt and ERK signaling in non-cancerous MRC5 lung cells. Whole cell lysates were prepared from MRC5 cells that were serum-deprived for 24h then treated with 1% wine for 24h. Cell lysates (20μg) were resolved by SDS-PAGE and immunoblotted with total- or phospho- specific antibodies against A) Akt or B) ERK. Insulin stimulated (100nM for 15min) L6 muscle cells were used as a positive control. A representative immunoblot is shown. The densitometry of the bands expressed in arbitrary units was measured using Image J software. Protein levels are expressed as a percentage of the control. Results represent mean ± SEM of 3 independent experiments. ***$p<0.001$ vs. control

Resveratrol-fortified wines do not effect cell proliferation in non-cancerous lung cells

To explore whether the anti-proliferative effects of red wine were specific to cancer cells, the normal lung fibroblast cell line MRC5 was used. Exposure of MRC5 cells to various concentrations (0.02, 0.5, 1, 2, 5%) of unfortified and fortified red wine did not result in significant inhibition of cell proliferation at any concentration used. Cell proliferation was not significantly inhibited even at 5% red wine (-RSV: 93.9±6.5%; +RSV: 90.0±2.9%; ++RSV: 89.7±5.6% of control) (Figure 5).

Resveratrol-fortified wines do not effect Akt or ERK signaling in non-cancerous lung cells

We examined phosphorylated and total Akt and ERK levels in MRC5 normal lung fibroblasts. Unlike cancerous A549 lung cells, non-cancerous MRC5 lung cells have low basal phosphorylated ERK and Akt levels. Akt phosphorylation was almost undetected. We used a sample of insulin treated L6 muscle cells as a positive control. Akt phosphorylation was clearly seen in these cells indicating that lack of the detection in MRC-5 cells was not due to a technical problem. Both fortified and unfortified red wines (1%) had no effect on total or phosphorylated levels of Akt or ERK (Figure 6A and 6B respectively). Similarly, no effect was seen using 0.02% wine (data not shown).

Discussion

Lung cancer and specifically NSCLC is presented in both smokers and non-smokers with high frequency, and is associated with poor prognosis. In addition, NSCLC develops resistance to radiation and chemotherapy and therefore finding ways to prevent and treat this disease is of major importance.

Wine consumption in moderation has been linked to reduced cardiovascular disease, and limited *in vitro* (Barron *et al.* 2014, Elattar & Virji 1998, Wallenborg *et al.* 2009) and *in vivo* (Martínez *et al.* 2005) studies suggest anticancer properties of wine. Red wine contains the polyphenol resveratrol which has been shown to inhibit cancer cell proliferation and survival by affecting various signaling cascades involved in these processes (Aluyen *et al.* 2012, Kubota *et al.* 2002, Tessitore *et al.* 2000, Yang *et al.* 2014). Based on this evidence, wine fortified with resveratrol has been produced and is marketed as having additional health benefits compared to wine without any

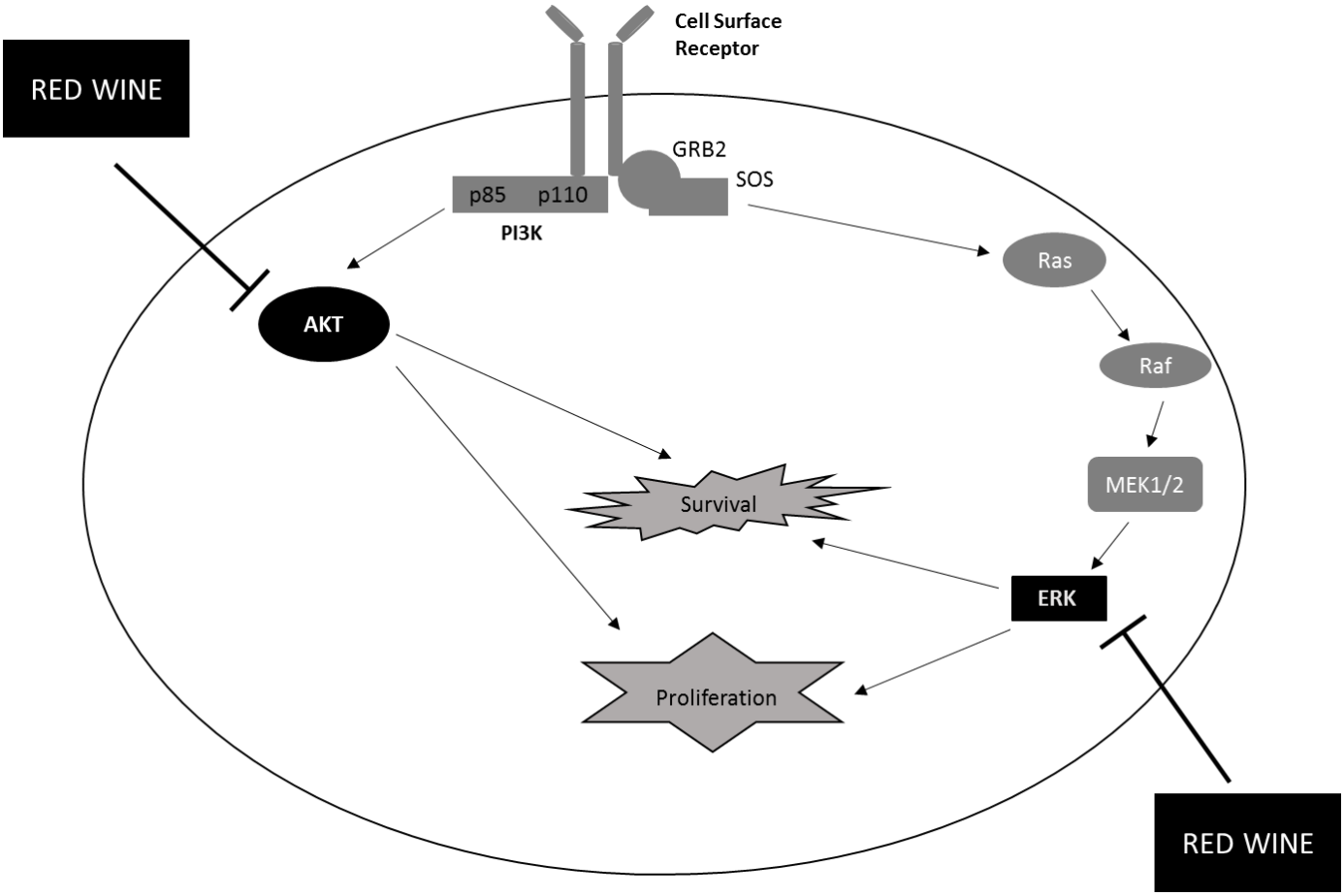

Figure 7. Proposed mechanism for anti-cancer activity of red wine in A549 lung cancer cells.

fortification. However, no studies examining the effects of fortified wine currently exist.

In the present study, we found a significant dose-dependent inhibition of cancer cell proliferation and survival by the Cabernet Sauvignon red wine. Wine concentrations as low as 0.02% significantly inhibited cell proliferation and survival. The data from the present study are in agreement with our previous study, where the red wines Pinot Noir and Cabernet Franc at 0.02% significantly inhibited survival of A549 lung cancer cells (Barron *et al.* 2014). Surprisingly, wine fortified with low or high resveratrol levels did not cause any further reduction in cancer cell proliferation and survival compared to unfortified wine. These data clearly indicate that components naturally present in red wine are sufficient to significantly reduce cancer cell survival. Although the antioxidant capacity of the wines used varied by around 16% (Table 1), this did not have any significant effect on cell survival, suggesting that once a certain level of antioxidant activity is reached it is sufficient to reduce cancer cell survival.

A study by Elattar & Virji (1998) found that the wine polyphenols resveratrol (50μM) and quercetin (10, 25 or 50μM) alone induced significant inhibition of human oral squamous cell carcinoma (SCC-25) growth and DNA synthesis, however a combination of the two compounds resulted in an increased inhibitory effect. Similarly, combined grape polyphenols resveratrol, quercetin and catechin at concentrations of 0.5, 5 or 20μM each significantly inhibited cell proliferation, induced apoptosis and enhanced cell cycle arrest in breast cancer cells and importantly, the combined treatment was more effective than treatment with any of the compounds alone (Castillo-Pichardo *et al.* 2009, Schlachterman *et al.* 2008). *In vivo* studies have also shown a greater inhibitory effect on tumor growth in nude mice xenografted with breast cancer cells using combined polyphenol treatment (resveratrol, quercetin, catechin; 5mg/Kg body weight each) (Castillo-Pichardo *et al.* 2009, Schlachterman *et al.* 2008). These data support the hypothesis that red wine components work synergistically to exert anticancer effects. This synergism may already be allowing wine to exert its maximal anticancer effects, and thus addition of resveratrol, or perhaps any other polyphenol, would not potentiate further these effects.

The inhibition of clonogenic cell survival by red wine clearly indicates that red wine has an anti-oncogenic potential, and in an *in vivo* setting such inhibition could lead to inability of a cancer cell to survive, form a colony and develop a tumor. Addition of red wine polyphenol extract in drinking water (100mg/Kg/day) of BALB/c nude mice implanted with colon carcinoma cells and Wistar rats injected with the carcinogen azoxymethane resulted in decreased tumor growth, onset and development of preneoplastic lesions (Walter *et al.* 2010). Furthermore, tumor vascularization, lung metastases and cell proliferation was decreased, and apoptosis was increased. These data clearly show that red wine polyphenols effectively reduce the development of colon carcinoma tumors *in vivo* and suggest anticancer and cancer preventive effects of wine that require further study.

The protein Akt has been established to play a major role in cell survival as its activation inhibits apoptosis and activates downstream the mTOR-p70S6K signaling cascade leading to enhanced cell proliferation (Manning & Cantley 2007). The A549 cells used in the present study express a mutant, activated Ras (K-Ras) gene, and are characterized by an enhanced Ras-PI3K-Akt cascade. We found a significant inhibition of Akt phosphorylation by red wine, and fortification with resveratrol did not result in any significant difference compared to unfortified wine, indicating that chemicals present naturally in red wine are sufficient to significantly inhibit Akt. The only other study to show inhibition of Akt by wine was performed by our lab (Barron *et al.* 2014). Resveratrol, at concentrations higher than those found naturally in wine, has been shown to inhibit Akt phosphorylation (100μM RSV) in human uterine cancer cells (Sexton *et al.* 2006) and radiation-induced Akt activation (2.5 or 5μM RSV) in human prostate cancer cells (Rashid *et al.* 2011). Furthermore, combined treatment with resveratrol, quercetin and catechin (5μM each) inhibited Akt activity by 50% compared to control in breast cancer cells (Castillo-Pichardo *et al.* 2009).

Activated Ras also leads to downstream activation of ERK and enhanced cell proliferation. We found an inhibition of ERK phosphorylation/activation and similar to survival, proliferation and Akt effects, fortified wine did not have any statistically different/enhanced effects compared to unfortified wine. The inhibition of ERK phosphorylation by red wine is in agreement with our previous study (Barron *et al.* 2014). Resveratrol has been shown to inhibit ERK phosphorylation in transgenic adenocarcinoma mouse prostate (TRAMP) males (Harper *et al.* 2007) and 50μM quercetin inhibited activation of ERK in human hepatoma cells (Granado-Serrano *et al.* 2006). Mice fed resveratrol (625mg/Kg) in their diet showed decreased ERK phosphorylation which contributed to an inhibition of proliferation, suppression of cancer development and a reduction in the incidence of poorly differentiated tumors (Harper *et al.* 2007), clearly indicating the potential for this polyphenol to have significant effects on this pathway. Figure 7 summarizes the in-

hibitory effects of red wine on Akt and ERK signaling cascades in cancer cells. These cascades are involved in cell survival and proliferation and future studies should examine if red wine has the same inhibitory effect on them *in vivo*.

Importantly, the results showed that fortified and unfortified red wine had no inhibitory effects on normal lung cell proliferation and did not alter the Akt or ERK signaling molecules in these cells, suggesting that wine selectively targets cancer cells and spares normal healthy cells. This finding suggests that moderate consumption of red wine may exert beneficial health effects by inhibiting cancer cell proliferation and survival without affecting healthy cells. However, further studies should be performed *in vivo* to support this claim.

Although the data presented here do not support the use of resveratrol fortified wines for additional anticancer effects, it should be noted that further research using *in vivo* animal cancer models should be performed. Resveratrol has been shown to have relatively low oral bioavailability in human studies (Smoliga & Blanchard 2014) and is known to be metabolized extensively in the body. Thus, increasing the concentration of resveratrol in wine may enhance its *in vivo* bioavailability and therefore enhance the health benefits.

In conclusion, the present study confirmed a significant inhibition of lung cancer cell survival and proliferation by red wine and an inhibition of Akt and ERK activation, key signaling molecules involved in cancer cell survival and proliferation. These findings were shown to be specific to cancer cells and were not seen in non-cancerous normal lung fibroblasts. To our knowledge this is the first study examining the effects of resveratrol-fortified wines at the cellular level. Although more research using animal models of cancer and lung cancer xenograft studies are required, the data presented here suggest that caution should be taken when making claims of "additional health benefits" regarding phenolic-fortified wine beverages.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

JM performed all experiments, data analysis and contributed to the manuscript preparation. NG produced the wines and performed the wine analysis as supervised by GP. ET was responsible for the conception and design of the study, data analysis, data presentation and manuscript preparation. GP assisted with manuscript preparation. All authors read and approved the final manuscript.

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