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Resveratrol-Induced Apoptosis is enhanced in low pH environments associated with cancer

Uzma Shamim¹, Sarmad Hanif², Abdulmajeed Albanyan², Frances W. J. Beck⁴, Bin Bao³, Zhiwei Wang³, Sanjeev Banerjee³, Fazlul H. Sarkar³, Ramzi M. Mohammad⁴, Sheikh M. Hadi¹, and Asfar S. Azmi^{3,*}

¹Department of Biochemistry, Faculty of Life Sciences, AMU, Aligarh-202002 (U.P.) INDIA

²Blood Transfusion Research Group, College of Applied Medical Sciences, King Saud University, Riyadh, Saudi Arabia

³Department of Pathology, Karmanos Cancer Institute, Wayne State University

⁴Department of Oncology, Karmanos Cancer Institute, Wayne State University

Abstract

Many critical factors such as hypoxia, nutrient deficiency, activation of glycolytic pathway/Warburg effect contribute to the observed low pH in tumors compared to normal tissue. Studies suggest that such tumor specific acidic environment can be exploited for the development of therapeutic strategies against cancer. Independent observations show reduction in pH of mammalian cells undergoing internucleosomal DNA fragmentation and apoptosis. As such, our group has extensively demonstrated that anticancer mechanisms of different plant polyphenols involve mobilization of endogenous copper and consequent internucleosomal DNA breakage. Copper is redox active metal, an essential component of chromatin and is sensitive to subtle pH changes in its microenvironment. Here we explored whether, acidic pH promotes growth inhibition, apoptosis and DNA damaging capacity of chemopreventive agent resveratrol. Our results reveal that growth inhibition and internucleosomal DNA fragmentation induced apoptosis in Capan-2 and Panc-28 pancreatic cancer cell lines (and not in normal HPDE cells) by resveratrol is enhanced at lower pH. Using comet assay, we further demonstrate that DNA breakage by resveratrol is enhanced with acidification. Membrane permeable copper specific chelator neocuproine (and not iron chelator orthophenanthroline) abrogated growth inhibition and apoptosis by resveratrol. Western blot results show enhanced activation of DNA laddering marker H2.aX by resveratrol at acidic pH that was reversed by neocuproine and not by orthophenanthroline. Our findings provide irrevocable proof that low pH environment can be turned into tumor weakness and assist in eradication of cancer cells by resveratrol.

Keywords

Warburg Effect; Acidic pH; Resveratrol; DNA breakage; Comet Assay; Apoptosis

1. Introduction

It is well recognized that epithelial tumors have lower pH compared to normal tissues due to a number of factors such as hypoxia, lack of vasculature and high rate of glycolysis followed

*To whom correspondence should be addressed: Asfar S. Azmi, Department of Pathology, Karmanos Cancer Institute, HWCRC building 732, 4100 John R, Detroit MI 48201. Phone: 313 576 8328; Fax: 313 576 838; asfar_azmi@wayne.edu; azmia@karmanos.org.

by lactic acid fermentation in the cytosol (also termed as Warburg effect) (Gerweck, 1998). As a consequence, a significant pH gradient difference exists between tumor and normal tissues (Gerweck and Seetharaman, 1996). It has been suggested that this pH gradient difference may be exploitable for the treatment of cancer by cytotoxic drugs, chemopreventive compounds and targeted therapies (Wike-Hooley et al, 1984). Several reports have supported the view that intracellular acidification in mammalian cells undergoing apoptosis may be a generalized feature (Barry and Eastman, 1992; Matsuyama and Reed, 2000). Indeed, such an acidification has been detected following exposure of cells to apoptotic stimuli as varied as UV irradiation, staurosporine, etoposide, arsenic, anti-Fas antibodies, growth factor deprivation and somatostatin (Matsuyama and Reed, 2000). Drug-induced apoptosis in several cancer cell lines has been shown to be mediated through the induction of endonucleases (primarily endonuclease II or DNase II) causing internucleosomal DNA fragmentation (Nagata et al, 2003). While activation of the DNase II requires intracellular acidification during cell death (Barry and Eastman, 1992; Barry and Eastman, 1993). Several other nucleases such as endonuclease G (Barry and Eastman, 1993) and DFF40 (Widlak et al, 2000) have also been identified as apoptotic endonucleases. Interestingly Czene et al., had shown that DNA fragmentation in human fibroblasts may also occur through copper mediated site specific DNA breakage and such DNA breakage was found to be enhanced by intracellular acidification (Czene et al, 1997). These studies strongly indicate that acidification if not critical is certainly an integral part of the mechanism involved in cells undergoing apoptosis.

Of particular interest is the observation that different plant-derived polyphenolic compounds such as resveratrol, Gallic acid and EGCG have been shown to induce internucleosomal DNA fragmentation in cancer cell lines. However, such DNA fragmentation was not observed in normal human epidermal keratinocytes (Clement et al, 1998; Inoue et al, 1995; Inoue et al, 2000). The hallmark of apoptosis is internucleosomal DNA fragmentation which distinguishes it from necrosis. Most clinically used anticancer drugs can activate late events of apoptosis (DNA degradation and morphological changes) and there are differences in essential signalling pathways between pharmacological cell death and physiological induction of programmed cell death (Smets, 1994). Results from studies in our laboratory have shown that flavonoids (Rahman et al, 1992), tannic acid and its structural constituent Gallic acid (Bhat and Hadi, 1994), curcumin (Ahsan et al, 1999), galocatechins (Azam et al, 2003; Malik et al, 2003) and resveratrol (Azmi et al, 2005; Ahmad et al, 2005) cause oxidative strand breakage in DNA as a single agent or in the presence of transition metal ions such as copper (Azmi et al, 2006). Copper is an important metal ion present in chromatin and is closely associated with DNA bases, particularly guanine (Geierstanger et al, 1991; Kagawa et al, 1991; Kagawa et al, 1989). It is also one of the most redox active of the various metal ions present in cells and can be modulated by subtle changes in pH of the intracellular and surrounding environment (Huang et al, 2004). Based on our own observations and those of others we had earlier proposed a hypothesis according to which, the anti-cancer mechanism of plant polyphenols involves the mobilization of endogenous copper ions as well as chromatin-bound copper which leads to a pro-oxidant DNA cleavage reaction (Hadi et al, 2007; Hadi et al, 2000). In the process of validating our hypothesis we had already shown that EGCG and resveratrol mobilize nuclear copper from human peripheral lymphocytes leading to DNA breakage (Shamim et al, 2008).

To date, a number of mechanisms have been proposed for the observed anticancer effects of different plant-derived compound polyphenols. However, research in the field has failed to find a concrete explanation on the selectivity of different natural agents that specifically targets cancers cell while sparing normal cells. The answer may lie in the inherent differences between the pH of cancer and normal tissue. Even though there is compelling evidence in support of the fact that intracellular acidification promotes apoptosis, no studies

to date have investigated the anticancer, chemopreventive or apoptotic effects of different plant- derived polyphenolic agents under different pH conditions, especially those conditions which mimic the acidic tumor environment. In this report, we used permeabilized human lymphocytes as well as epithelial pancreatic cancer cell lines (models that are well known to lack tumor vasculature and mimic the hypoxic, low pH tumor environment), to demonstrate that acidification enhances resveratrol-induced cellular DNA break and apoptosis. These studies demonstrate for the first time that low tumor or tumor environmental pH can be turned into a cancer cell weakness on exposure to resveratrol system and may explain the observed selective effects of certain chemopreventive dietary polyphenols against cancer, but not normal cells.

2. Materials and Methods

2.1 Materials, Cell Lines and Culture Media

Resveratrol, neocuproine, orthophenanthroline, bathocuproine disulphonate, superoxide dismutase (SOD), agarose, low melting point agarose (LMPA), RPMI 1640, Triton X-100, Trypan blue, Histopaque1077 and phosphate buffered saline (PBS) Ca^{2+} and Mg^{2+} free were purchased from Sigma (St. Louis, MO). All other chemicals were of analytical grade. Resveratrol was dissolved in DMSO before use as a stock of 10 mM solution. Resveratrol in the presence of the buffers mentioned and at the concentrations specified, remained in solution throughout the incubation periods and did not lead to any appreciable change in the pH of the incubation media. The human pancreatic cancer cell lines Capan-2, Panc-28 and HPDE were obtained from American Type Culture Collection (Rockville, MD). Cell lines were maintained in continuous exponential growth by twice a week passage in McCoy's 5A (for Capan-2) and DMEM modified Eagles medium (for Panc-28) supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 10 mg/ml streptomycin. HPDE cells were grown in keratinocyte serum-free (KSF) medium. In order to maintain a stable pH, culture media was supplemented with 5% Hepes (GIBCO). Cells were grown in standard humidified incubator containing 5% CO_2 in air at 37°C. Each cell line was routinely split before attaining 70–80% confluence.

2.2. Culture media pH adjustments

pH of culture media was adjusted using sterile HCL (Fischer Scientific USA) followed by vacuum filtration prior to use. All evaluations of pH were performed in sterile environment to avoid contamination. pH was recorded at different time points for changes (prior to start of treatment, and at the end of incubation period). There were no appreciable changes in pH of the culture media due to the addition of test agents or with incubation time.

2.3. Cell growth inhibition by Trypan blue and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT)

Capan-2, Panc-28 and HPDE cells were seeded at a density of 3×10^3 cells per well in 96-well micro titer culture plates. After overnight incubation, medium was removed and replaced with fresh medium containing resveratrol (50 μM) diluted from a 10 mMol/L stock. On completion of 72 hours of incubation, viability was assessed adding 50 μL of Trypan Blue solution (0.4% in PBS) in culture medium. After 1–2 min the number of dead cells, which retained the dye, was compared to the total number to calculate the viability percentage. MTT assay was performed by adding 20 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/mL in PBS) to each well and incubated further for 2 hours. Upon termination, the supernatant was aspirated and the MTT formazan formed by metabolically viable cells was dissolved in 100 μL of DMSO. The plates were gently rocked for 30 minutes on a gyratory shaker, and absorbance was measured at 595 nm using a plate reader (TECAN, Durham, NC).

2.4. Quantification of apoptosis by Histone DNA ELISA and Annexin V FITC Assay

The Cell Apoptosis ELISA Detection Kit (Roche, Life Sciences) was used to detect apoptosis in Capan-2, Panc-28 and HPDE cells with different treatments according to the manufacturer's protocol. This assay can be used for relative quantification of histone-complexed DNA fragments (mono- and oligonucleosomes) from the cytoplasm of cells after the induction of apoptosis or when released from necrotic cells. Since the assay does not require prelabeling of cells, it can detect internucleosomal degradation of genomic DNA during apoptosis even in cells that do not proliferate *in vitro* (for example, freshly isolated lymphocytes). All procedures were performed according to our previously published protocol (Azmi et al, 2011).

2.5 Isolation of lymphocytes

Heparinized blood samples (2 ml) from a single healthy donor was obtained by venepuncture and diluted suitably in Ca^{2+} and Mg^{2+} free PBS. Lymphocytes were isolated from blood using Histopaque 1077 (Sigma) and the cells were finally suspended in RPMI 1640.

2.6. Viability assessment of whole cells

Cell viability (lymphocytes as well as cancer cells) were checked for their viability at the beginning and after the end of the incubation period using Trypan Blue Exclusion Test.

2.7. Preparation of permeabilized lymphocytes

Permeabilized lymphocytes were prepared as described by Czene et al. (Czene et al, 1997) with slight modifications. Lymphocytes isolated from 2 ml blood were diluted in RPMI 1640 to a concentration of 2×10^5 cells/2 ml. Approximately 10,000 cells were mixed with 75 μl of pre-warmed low melting point Agarose (LMPA) (Sigma St Louis MO) in PBS and immediately applied to a frosted microscopic slide layered with 1% standard agarose in PBS. The agarose coated slides containing cells were allowed to solidify at 4°C for 10 min. The lymphocytes on the slides were then exposed to the permeabilization solution (0.5 % triton X-100 in 0.004 M Tris-HCl, pH 7.4) for 10 min on ice. The resulting permeabilized cells consisted of only cell organelles attached to the residual cytoskeleton.

2.8. Treatment of permeabilized lymphocytes with resveratrol and evaluation of DNA breakage by comet assay

For treatment, slides containing the permeabilized lymphocytes were transferred to a rectangular dish ($8\text{cm} \times 3\text{cm} \times 5\text{mm}$) containing incubation media with or without resveratrol or other additions as specified in legends to figures and tables. The slides were incubated at 37°C for desired time periods (1 hr for intact cells / 30 min for permeabilized cells), after which they were washed twice by placing them in 0.4 M phosphate buffer (pH 7.5) for 5 min at room temperature. DNA unwinding and expression of alkali labile sites was done by leaving the slides in the high pH electrophoresis buffer (1 mM EDTA, 300 mM NaOH, pH > 13 prepared in PBS) at 4°C for 30 min. Subsequently, the electrophoresis, neutralization and staining of the slides was carried out as described earlier (Azmi et al, 2005). Comet images were observed at $100\times$ magnification with a fluorescence microscope (Olympus CX41) and COHU 4910 (equipped with a 510–560 nm excitation and 590 nm barrier filters) integrated CC camera. 50 images were randomly selected from each sample and their lengths (diameter of the nucleus plus migrated DNA) were measured on the screen as automatically generated by Komet 5.5 image analysis system of Kinetic Imaging, Liverpool, UK.

2.9. Protein Extraction and Western blot Analysis

Capan-2 and Panc-28 were plated and allowed to attach for 36 hours. Resveratrol (50 μ M) was directly added to cell cultures with different pH (pH 7.5, 7.0 and 6.5). Control cells were incubated in the medium containing an equivalent concentration of DMSO. In another set, cells were exposed to resveratrol in After a 24 hours incubation, cells were harvested in PBS and whole cell lysate was prepared and western blot was performed as described in our previous publication (Azmi et al, 2011). Antibody against H2.aX was purchased from Cell Signaling (Danvers, USA) while β -actin and all secondary antibodies were obtained from Sigma (St. Louis USA)

2.10. Statistics

The statistical analysis on Comet Assay data was performed as described by Tice et al. (Tice et al, 2000) and is expressed as Mean $\pm$ S.E.M. of three independent experiments. A student's t-test was used to examine the significance of statistical differences in all analyses (MTT, Histone DNA ELISA and Annexin V FITC apoptosis). Analysis of variance was performed using ANOVA. P values < 0.05 were considered statistically significant.

3. Results

3.1. Resveratrol shows enhanced growth inhibitory and apoptotic potential in PDAC cells at lower pH

In order to investigate the effect of pH on growth inhibition by resveratrol, MTT assay was performed on Capan-2, Panc28 (cancer) and HPDE (normal) cells at different pH conditions (pH 7.5, 7.0 and 6.5). There was no statistically significant basal level growth inhibition in the cancer or normal cells grown at the tested pH range for 72 hrs (Figure 1A). In the presence of resveratrol a statistically significant enhanced growth inhibition at lower pH was observed only in Capan-2 and Panc-28 and not in HPDE cells (Figure 1B). Viability was also assessed using trypan blue assay that showed similar trend that was again specific for cancer cells only (cell death pH 6.5>7.0>7.5). Further, the apoptotic potential of resveratrol was investigated under different pH conditions using an internucleosomal DNA fragmentation specific Histone/DNA ELISA assay. As can be seen from results of Figure 1C, cancer cell lines demonstrate enhanced apoptosis at pHs lower than 7.5 (with no statistically significant effect is seen in normal counterpart HPDE cells). More significantly, as Histone/DNA ELISA assay provides direct evidence of endonuclear DNA fragmentation, this certainly validates our hypothesis regarding the critical role of pH in resveratrol-induced apoptosis. These results further corroborates that at lower pH resveratrol has enhanced growth inhibitory potential in cancer cell lines of a tumor model that is well recognized to lack proper vasculature.

3.2. Effect of metal chelators on growth inhibition by resveratrol

In order to verify whether growth inhibition by resveratrol was the result on cellular copper localization, a membrane permeable copper chelator neocuproine was used. As can be seen from results of Figure 2, growth inhibition by resveratrol was abolished by progressively increasing concentrations of neocuproine at two different time points (72 and 96 hrs). Orthophenanthroline, a membrane permeable iron chelator, did show any statistically significant effect. These dose and time dependent effects observed specifically with neocuproine strongly demonstrate that indeed copper is involved in resveratrol mediated growth inhibition. TPEN a zinc specific chelator had no effect as well (data not shown). Additionally, the effect of metal chelation on apoptosis was also investigated using histone DNA ELISA in the presence of neocuproine or orthophenanthroline. Similar to MTT results presented above, only neocuproine showed statistically significant suppressive effects on

resveratrol mediated apoptosis (Figure 3). Cumulatively, our results firmly establish that resveratrol induced growth inhibition and apoptosis is resultant of copper mobilization.

3.3 Low pH enhances induction of internucleosomal fragmentation marker H2.aX by resveratrol

In order to explore whether low pH facilitates induction of internucleosomal fragmentation (DNA laddering) marker, we analyzed the effect of resveratrol on activation of the histone variant H2.aX that has been shown to be required for DNA ladder formation both *in vivo* and *in vitro* (Lu et al, 2006). As can be seen from results of Figure 4A, western blot analysis using an antibody that recognized H2.aX indicates that resveratrol mediated induction of H2.aX in both Capan-2 and Panc-28 was enhanced at low pH. Of paramount significance is the observation that H2.aX induction by resveratrol at pH 6.5 was abrogated by neocuproine and not by orthophenanthroline (Figure 4B) in both cell lines. Taken together, our growth inhibition, apoptosis and comet assay results and expression profiling data obtained through western blot provides irrevocable proof that resveratrol mediated anti-cancer effects are stimulated at acidic pH in PDAC cell lines.

3.4. Effect of metal-specific chelators on resveratrol-induced DNA breakage in intact and permeabilized lymphocytes at pH 7.5

In a previous report, we had studied resveratrol-induced cellular DNA breakage in a permeabilized cellular system which allows direct interaction of resveratrol with nuclei by eliminating the cell membrane and cytoplasmic barriers [28]. It was shown that considerably greater DNA degradation was observed in permeabilized cells as compared with intact lymphocytes. In the experiment shown in Table 1, we have used various metal-specific chelators, which selectively bind to copper, iron and zinc, to study their effect on resveratrol-induced DNA degradation in intact lymphocytes as well as in permeabilized cells. In intact cells, a clear inhibition of DNA degradation was observed with neocuproine (a cell membrane permeable Cu(I) specific chelator). However, in whole lymphocytes, no such inhibition was seen with bathocuproine (a water soluble membrane-impermeable analogue of neocuproine), desferrioxamine mesylate (a Fe(II) specific chelator) or histidine (a zinc specific chelator). This is in contrast with the results found using permeabilized lymphocytes, as both neocuproine and bathocuproine were found to inhibit the DNA breakage while iron and zinc chelators were still ineffective in causing such inhibition. Bathocuproine disulphonic acid, which is impermeable to intact cell membranes, can freely traverse through permeabilized cells to directly interact with the cell nuclei. Furthermore, it should be noted that the nuclear pore complex is permeable to small molecules. These results suggest that, irrespective of whether intact lymphocytes or permeabilized lymphocytes are used, resveratrol specifically mobilizes chromatin bound copper, and not other trace metals, leading to oxidative DNA breakage.

3.6 Effect of chelators on resveratrol-induced DNA breakage at pH 6.5 in permeabilized lymphocytes

The above results suggest that the acidic milieu may facilitate the mobilization of chromatin bound copper by resveratrol. This leads to an enhanced degree of DNA breakage by resveratrol at acidic pH. It is known that copper and zinc are the major metal ions present in the nucleus (Olivares and Uauy, 1996). Therefore, the role of major intracellular metals, including copper, in the observed DNA breakage, was assessed using their specific chelators under acidic conditions resembling those found in aggressively growing cancer cells. The results in Table II clearly again, indicate the involvement of copper but not of iron or zinc in the induction of a greater degree of DNA breakage by resveratrol at lower pH. Due to extensive DNA damage at acidic pH we scaled down resveratrol to 20 μ M instead of 50 μ M as used in Table 1. Nevertheless we observe statistically significant suppression of DNA

damage by copper specific chelator. The results are also in concurrence with the observation made by Czene and group where 1,10 phenanthroline but not desferrioxamine mesylate inhibited DNA breakage induced by acidic pH (Czene et al, 2002).

4. Discussion

Studies presented in this paper are the first to show that the DNA damage and apoptosis inducing capacity of resveratrol is stimulated at acidic pH. These findings may explain for the selective toxicity of resveratrol towards cancer cells and has implications in the treatment of epithelial tumors that are less vascularised, hypoxic and are recognized to have low pH microenvironment. In essence, these results support the idea that Warburg effect can be utilized to develop cancer specific therapies.

The development of effective therapy for malignant disease has been hindered by lack of consistent differences between cancer and normal tissue. Thus, unlike antibiotic treatment for bacteria, it has been difficult to develop therapeutic strategies that have major toxic effects against tumors but spare normal tissue. One major difference between solid tumors and surrounding tissue is the nutrition and metabolic environment. Over the years it has been well established that most solid tumors have low vasculature that restricts proper nutrient supply leading to deficiency of oxygen (hypoxia) (Boyle and Travers, 2006). Hypoxia in turn leads to reduction in tumor pH and a possible explanation for the observed phenomena can also be inferred from the *Warburg Effect*. According to Warburg effect, oxygen's relationship to the pH of cancer cell's internal environment is directly correlated. Since fermentation is a major metabolic pathway, cancer cells maintain a lower pH, as low as 6.0, due to lactic acid production and elevated CO₂ (Parkins et al, 1997). The concept that cancer cells switch to glycolysis has become widely accepted, even if it is not seen as the cause of cancer. Some suggest that the Warburg phenomenon could be used to develop anticancer drugs (Kim and Dang, 2006).

Over the last decade our laboratory has demonstrated that the anticancer mechanism of different dietary polyphenolic compounds can be attributed to their pro-oxidant and DNA damaging effects (Hadi et al, 2000). Our hypothesis suggests these antioxidants can mobilize cellular copper (elevated in tumor tissue and through a redox mechanism inducing reactive oxygen species leading to oxidative DNA breakage and internucleosomal fragmentation. We have previously provided evidence to show that this is an alternative, non-enzymatic and copper-dependent pathway for the cytotoxic action of dietary compounds that are capable of mobilizing and reducing endogenous copper (Azmi et al, 2006). As such, this is independent of Fas and mitochondria mediated programmed cell death. Supporting our hypothesis, results of several studies have indicated that although apoptosis induction by several polyphenols and other anticancer agents is independent of caspases and mitochondria, it is accompanied by an increase in intracellular levels of ROS (Noda et al, 2007). An important component of our hypothesis is that plant polyphenols possessing anticancer and apoptosis-inducing properties, mobilize chromatin bound copper which undergoes redox cycling and further leads to the formation of ROS such as the hydroxyl radical. This is also in concurrence with the idea that, because of its extreme reactivity, the hydroxyl radical must be produced in the vicinity of DNA in order to cause its cleavage. Indeed, such a common mechanism better explains the anticancer effects of polyphenols with diverse chemical structures and the preferential cytotoxicity towards cancer cells.

This now begs the important question regarding the relationship between our pro-oxidant hypothesis, Warburg Effect and low tumor pH? The answer lies in the results presented where we convincingly demonstrate that cellular DNA breakage induced by resveratrol (and

possibly by other plant polyphenols) is stimulated at acidic pH. It is well recognized that copper is an important metal ion present in chromatin and is closely associated with DNA bases, particularly guanine (Kagawa et al, 1991). Earlier studies have shown that at low pH, protonation on N7 of guanine may cause rotation of the base out of the helix (Courtois et al, 1969) thus possibly facilitating the mobilization of copper by metal chelating drugs (in our case by resveratrol). Of paramount significance is the finding that membrane permeable and copper specific chelators abrogate DNA breakage by resveratrol. Taken together these results validate our hypothesis that low pH induced copper availability is integral to the effect of resveratrol and may explain for the selectivity of resveratrol to induce internucleosomal DNA fragmentation as copper mobilization by resveratrol is facilitated at acidic pH.

Taking a clinical perspective, we analyzed the effect of resveratrol on pancreatic cancer cell lines that represent epithelial tumors (mimicking hypoxic low pH environment). Growth inhibition and viability assay results in these cells showed enhanced sensitivity to resveratrol at lower pH (Figure 1 and 2). Multiple apoptosis assay evaluations (Histone DNA ELISA specific for internucleosomal DNA fragmentation and Annexin V FITC) indicated a similar trend with greater apoptosis in Capan-2, Panc-28 and HPDE cells at pH 6.5 and pH 7.0 compared to that observed at pH 7.5. Although, histone DNA ELISA is a direct proof of internucleosomal fragmentation we further analyzed DNA laddering markers H2.aX at different pH conditions in two cell lines. In line with our growth inhibition, viability and apoptosis results, induction of H2.aX expression by resveratrol was found to be greater at low pH. Most significantly, the expression of H2.aX was abolished by neocuproine and not by orthophenanthroline is further suggestive of the specific role of copper in this reaction. Further, using a sensitive DNA strand breakage recognizing comet assay in both whole cells and permeabilized lymphocytes, we proved that indeed at low pH there is enhanced strand breakage that could be specifically reverted by copper specific chelators only. One can argue that the doses required for resveratrol to induce DNA breakage is too high to be achieved physiologically. However, it should be noted that tumors have high copper content that would facilitate mobilization by resveratrol even at low (physiological) concentrations leading to tumor specific DNA breakage and ultimately apoptosis.

Based on our results, we propose a model in which at physiological pH Cu(II) attached to guanine base is buried and this prevents interaction with resveratrol. On the other hand in low pH environment, due to rotation of guanine base, Cu(II) is exposed that allows resveratrol mediated mobilization (Schematic Figure 5). Our previous reports have verified that copper chelation by resveratrol results in Cu(II) reduction to Cu(I) and Fenton re-oxidation of Cu(I) to Cu(II) results in generation of ROS leading to oxidative DNA breakage. We are currently exploring the molecular mechanism behind the observed up-regulation of H2.aX, which we believe is a secondary consequence of ROS mediated DNA damage and internucleosomal fragmentation.

In conclusion, we propose that low pH environment of tumor due to multiple factors such as reduced vasculature, nutrient deprivation and hypoxia in the backdrop of Warburg Effect can be converted to cancer cell weakness by resveratrol. This can be the explanation for the observed selective anti-cancer effects of different plant polyphenols against cancer cell sparing normal and in essence is a proof of concept that Warburg effect can be utilized for the development of anti-tumor strategies.

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Figure 1. Low pH enhances growth inhibition and apoptosis by Resveratrol in Pancreatic Cancer Cells sparing normal cells

Capan-2 and Panc-28 (for PDAC) and HPDE (normal epithelial cells) were grown in 96 well plates at a density of 3×10^3 cells/well. After 24 hrs cells were exposed to either DMSO or Resveratrol (50 μ M) for 72 hrs. [A] Growth inhibition was evaluated by MTT assay, [B] Viability by Trypan Blue exclusion assay and [C] Apoptosis was evaluated by Histone DNA ELISA assay as described in methods section. Note enhanced growth inhibition and apoptosis at pH 6.5 and pH 7.0 compared to that observed at pH 7.5 (Shaded bars). Values represent mean of three independent experiments. ** $p < 0.01$. Normal HPDE cells did not respond to resveratrol treatment. In the absence of resveratrol, there was no statistically significant growth inhibition in cells grown in both normal and cancer cells at different pH environments (White bars). Procedure for pH adjustment is given in Methods Section. Note: no appreciable change in growth pattern of cells at different pH conditions.

Figure 2. Effect of Metal Chelators on Resveratrol Mediated Growth inhibition

Capan-2 and Panc28 cells were exposed to resveratrol ($50 \mu\text{M}$) at pH 6.5 in the presence increasing concentrations of membrane permeable copper chelator neocuproine ($0-75 \mu\text{M}$) or iron chelator orthophenanthroline ($0-75 \mu\text{M}$) for 72 or 96 hrs. [A] Growth inhibition was evaluated using MTT. Note growth inhibition by resveratrol is abrogated by neocuproine and not by orthophenanthroline. All values represent mean of three independent experiments. * represents $p < 0.05$ and ** represents $p < 0.01$

Figure 3. Effect of Metal Chelators on Resveratrol Mediated Apoptosis

Capan-2 and Panc28 cells were exposed to resveratrol ($50 \mu\text{M}$) at pH 6.5 in the presence increasing concentrations of membrane permeable copper chelator neocuproine ($0-75 \mu\text{M}$) or iron chelator orthophenanthroline ($0-75 \mu\text{M}$) for 72 or 96 hrs. Apoptosis by histone DNA ELISA assay. Note: growth inhibition and apoptosis by resveratrol is abrogated by neocuproine and not by orthophenanthroline. All values represent mean of three independent experiments. * represents $p < 0.05$ and ** represents $p < 0.01$

Figure 4. Resveratrol mediated DNA damage marker H2.aX expression is enhanced at lower pH Capan-2 and Panc-28 cells grown in different pH environment were exposed to Resveratrol in 100 mm Petri dishes. After 24 hrs, cells were harvested and protein according to protocols described in material and methods. Western blotting was done according to standard protocols. Membranes were probed with H2.aX antibody and corresponding secondary antibody. β-actin protein was used as loading control and is shown for each blot. [A] pH dependent enhancement of H2.aX (6.5>7.0>7.5) and [B] copper chelation by neocuproine suppresses H2.aX activation and no effect is observed on orthophenanthroline (OP) treatment. Blot representative of three independent experiments.

Figure 5. DNA strand breakage evaluation by resveratrol at different pH using Comet Assay Isolated lymphocytes were exposed to resveratrol (50 μ M) for 2 hrs in three pH conditions (pH 7.5, 6.5 and 5.5) and cell were processed using Comet assay as described in Material and Methods. Comet tail length (μ meters) is plotted as a function of DNA breakage in permeabilized lymphocytes. White bars are control tail lengths at different pH while black bars are tail lengths after resveratrol treatment. Note enhanced tail length indicative of DNA breakage at lower pH. All points represent mean of three independent experiments. Error bars denote $\pm$ SEM. P value < 0.05 and significant when compared to control.

Figure 6. Resveratrol exploits the pH differences between normal and tumor tissue (a mechanistic summary)

Copper is an essential component of DNA and is found associated to guanine bases. In normal tissue (and at physiological pH), copper associated to guanine is oriented in such a manner so as to prevent chelation by agents such as resveratrol (resveratrol depicted as ball and stick model). In low pH environment, due to rotation of guanine base, copper is exposed and can be mobilized by resveratrol to form a ternary complex leading to reduction of Cu(II) to Cu(I) and consequent oxidation of resveratrol. Fenton type re-oxidation of Cu(I) to Cu(II) leads to production of ROS and consequent DNA breakage.

Table I

Effect of metal-specific chelators on resveratrol-induced DNA breakage in intact and permeabilized lymphocytes at pH 7.5

Resveratrol (50 μ M)	Chelator (100 μ M)	Whole Lymphocytes		Permeabilized Lymphocytes	
		Control Tail length (μ m)	Tail Length (μ m)	Control Tail length (μ m)	Tail Length (μ m)
Resveratrol	-	13.68 $\pm$ 1.03 ^a	-	30.32 $\pm$ 2.73 ^b	-
Resveratrol	Neocuproine	-	6.03 $\pm$ 0.51	-	17.27 $\pm$ 1.14
Resveratrol	Bathocuproine	-	13.6 $\pm$ 1.02	-	20.05 $\pm$ 1.26
Resveratrol	Histidine	-	13.44 $\pm$ 1.01	-	30.16 $\pm$ 2.81
Resveratrol	Desferrioxamine mesylate	-	13.3 $\pm$ 1.01	-	29.88 $\pm$ 2.56

Data represent as Mean $\pm$ S.E.M of three independent experiments

^a p<0.05 as compared with untreated whole lymphocytes (Tail length = 1.30 $\pm$ 0.13)

^b p<0.05 as compared with untreated permeabilized lymphocytes (Tail length = 2.72 $\pm$ 0.17)

Table II

Effect of chelators on resveratrol-induced DNA breakage at pH 6.5 in permeabilized lymphocytes

Dose	Tail length (μm)
Untreated	9.87 ± 1.28
Resveratrol (20 μM)	$42.13 \pm 2.46^{\#}$
+Neocuproine (1mM)	$24.49 \pm 1.93^*$
+Bathocuproine (1mM)	$20.18 \pm 1.72^*$
+Desferrioxamine mesylate (1mM)	$41.78 \pm 2.38^*$
+ Histidine (1mM)	$41.26 \pm 2.31^*$

All values represent Mean $\pm$ S.E.M. of three independent experiments.

* P values < 0.05

when compared to control.