DISSERTATION

IN-VIVO INVESTIGATION OF RESVERATROL AS A PREVENTIVE FOR RADIATION-INDUCED ACUTE MYELOID LEUKEMIA

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY RONALD E. CARSTEN ENTITLED *IN-VIVO* INVESTIGATION OF RESVERATROL AS A PREVENTIVE FOR RADIATION-INDUCED ACUTE MYELOID LEUKEMIA BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

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Resveratrol has been shown to have cancer preventive properties. It modulates a wide range of molecular targets including those involved with induction of apoptosis and cell cycle arrest in a concentration dependent fashion. This study was designed to investigate resveratrol's ability to reduce radiation-induced chromosome aberrations and PU.1 gene loss in mouse bone marrow cells. Loss of 1 PU.1 gene and missense mutation of the remaining allele leads to acute myeloid leukemia in CBA mice.

Male CBA mice were divided into groups of 10 mice. Control groups consisted of no treatment, resveratrol, muscadine grape extract (MGE), and radiation with end points of 1, 7, and 30 days post-irradiation. Experimental groups were administered resveratrol starting 2 days before irradiation with bone marrow collected at 1 and 30 days; resveratrol initiated 2 hours (as a single dose or continued to the end point) or started 2 days (continued to the end point) after irradiation with collection at 1, 7, and 30 days; and resveratrol or MGE at specified doses starting 2 days before irradiation with bone marrow collection1 day after radiation exposure. Cytogenic evaluation and fluorescence *in situ* hybridization was performed.

Resveratrol significantly ($p<0.05$) reduced mean chromosome aberrations at all end points when initiated before or after irradiation. The optimal dose for reducing chromosome aberrations at day 1 for resveratrol alone was 6.25-25 mg/kg and for

resveratrol in MGE was 2.10-7.13 μg/kg. Loss of the PU.1 gene was significantly ($p<0.0001$) reduced at 1 or 30 days with resveratrol and MGE administration.

When initiated pre-irradiation, resveratrol reduced chromosome aberrations and PU.1 loss at 1 and 30 days, indicating radioprotection. Cancer prevention was implied when resveratrol was initiated after irradiation resulting in reduced chromosome aberrations at 1, 7, and 30 days and reduced PU.1 gene loss at 1 and 30 days. Resveratrol pre-irradiation was not more beneficial than a single dose 2 hours post-irradiation ($p>0.05$) at 30 days; resveratrol initiated pre-irradiation was more effective ($p<0.05$) than continued resveratrol started 2 days post-irradiation at 30 days. The μg/kg dose of resveratrol found in MGE was significantly more effective than the mg/kg dose of resveratrol.

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Acknowledgements

Our life journey is punctuated by interactions with people and events that can represent turning points that shape our decisions and mold our lives. We are fortunate to have the opportunity to encounter people that have positive and lasting influences on our lives. These people stand out in our memories and we are grateful for the journey that we shared. One such individual was my brother, Robert S. Carsten (1955-2001). I have almost no childhood memory that does not include him. He was a person that treated the least of us with the utmost respect, he defended those who couldn't defend themselves, he treated everyone with fairness, he worked hard every day, he persevered with all his strength in all that he did, he never complained while accepting life as it came, and he helped all that needed help without expectation of return. These are the lessons that he taught me as we discovered life and experienced together the adventures that life offered. I watched this man face great challenges as he battled terminal cancer, beat the odds to attend his daughter's wedding and see the birth of his grandson, and never complain about the unfairness of his situation or the disabling pain that he experienced. It is this last shared experience that finalized my decision to return to the university and pursue this Doctor of Philosophy degree. This is my humble contribution, in memory of a simple man that inspired many to be better people, that I dedicate my efforts to change the course of how cancer patients are managed.

Without the constant support of my wife and friend, Toni, and my children, Joseph and Christine, I could not have left my veterinary practice to begin this journey and to see it through to the end. They are each in their own way an inspiration and have shown me facets of life that I could not have otherwise seen. I am especially proud of the

individuals that my children have become – they exemplify all the ideals that I hold in high regard and they have become strong and resilient people capable of achieving anything that they choose.

When I made the decision to leave my veterinary practice to return to Colorado State University, it was my staff and clients at Birch Tree Animal Hospital that without hesitation supported my goals and continuing efforts to make a broader contribution to our community and the veterinary profession. Their support has always been an inspiration for me even as it drove me to constantly search for appropriate therapeutic options and case management approaches that could optimize their companion's quality of life.

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Introduction

Resveratrol (*trans*-3,5,4'-trihydroxystilbene) has been widely studied following its identification as a cardioprotectant in 1992 (1). Since then, resveratrol has been shown to have cancer preventive, anticancer, anti-inflammatory, and anti-aging effects. These effects have been studied in a wide variety of cell lines, tissue culture models, and ex-vivo models using numerous resveratrol concentrations with different resveratrol contact times. In addition, resveratrol has been studied in a smaller number of *in vivo* models using a wide range of doses administered orally or intraperitoneally. Results of these studies show that resveratrol affects a significant number of molecular targets and signaling pathways in a dose and contact time dependent fashion. Examples of molecular targets that are modulated by resveratrol include the following: retinoblastoma protein, cyclins A, B1, and D, Cdk2, p21, p53, TNFα, IL-1β, Bax, Bad, Bcl-2, IL-6, survivin, caspases 2, 3, 8, and 9, Cox-2, iNOS, ICAM-1, VEGF, protein kinase C, MAPKs, Erk1/2, CYP1A1, SIRT1, ribonucleotide reductase, and DNA polymerase (2,3). Interestingly, cancer cell lines appear to be more sensitive than normal cells to the impacts of resveratrol on molecular targets. Further, effects on normal cells appear to be reversible in a concentration dependent manner.

As a phytoalexin, resveratrol is produced in the plant to act as an antimicrobial agent against fungal organisms like *Botrytis cinerea* infection (4). The stilbene polyphenol, resveratrol, is also produced in response to environmental stresses such as dry conditions, and UV irradiation. It has been identified in at least 72 plant species including grapes, peanuts, blueberries, bilberries, cranberries, lingonberries, and the weed *Polygonum cuspidatum* (5-12). Historically, resveratrol was first identified in 1940 by Takaoka in the roots of a *Veratrum* species initially misidentified as *V. grandiflorum* and later

believed to be *V. album var. L grandiflorum* (13). The origin of the name resveratrol is reportedly derived from the Latin word 'res' meaning "which comes from" the plant 'veratrum' and 'ol' indicating the presence of an alcohol moiety (14). In 1963, Nonomura described resveratrol in the roots of *Polygonum cuspidatum* (15). Interestingly, *P. cuspidatum* has been a constituent of the Chinese and Japanese traditional medicine remedy, Ko-jo-kon, for over 2,000 years. This medicinal remedy has historically been used as a circulatory tonic, for hyperlipidemic diseases, for liver diseases, suppurative dermatitis, arteriosclerosis, allergies, and inflammatory diseases (3, 15,16). Resveratol was next described in grapes in 1976 by Langcake (17) and in wine in 1992 by Siemann (16). Also, in 1992, Renaud identified a correlation between the consumption of wine in France and a reduced incidence of coronary heart disease in the face of a diet high in saturated fat (1). Another fascinating historical observation is in regard to darakchasava, an Ayurvedic medicinal remedy that is mainly composed of dried grape berries that are boiled and fermented. This remedy has been in use for over 4,500 years as a general health tonic and a cardiotonic (3, 18). In 2003, resveratrol was reported to activate sirtuin deacetylases and extend the life span of *Saccharomyces cerevisiae* (19). Since that time, resveratrol has been shown to extend lifespan in *Caenorhabditis elegans, Drosophila melanogaster,* and *Nothobranchius furzeri* (5). Coincidentally, grapes are a component of the Ayurvedic medicinal rasyana tonics which have a long history of use as longevity tonics (20). Historical use of plants containing resveratrol is of interest because the traditional medicinal applications have significantly predated current applications and provide insights into future directions for research.

When reviewing the research on resveratrol there are a number of important observations to consider. First, the effects of resveratrol are concentration dependent. For example, in the acute myeloid leukemia cell lines OCIM2 and OCI/AML3, colony forming growth was suppressed in a concentration dependent manner with cell growth reduced by 60% with 20 μM resveratrol and completely stopped with 75 μM. In addition, cell cycle distribution in OCIM2 cells was altered in a concentration dependent fashion after 6 hours of incubation with resveratrol with an S phase accumulation of 25.50% (0 μM), 28.43% (10 μM), 53.70% (25 μM), and 64.61% (50 μM) (21). Second, the effects of resveratrol are contact time dependent. For example, progressive inhibition of human myeloma cells (IM9) and Burkitt's lymphoma cells (HS-sultan) was shown following exposure to 30 μM resveratrol for 0, 6, 12, 24, and 48 hours (22). Third, the effects of resveratrol are cell line dependent. For, example, resveratrol inhibited proliferation of 4 leukemia cells lines (mouse 32Dp210 and L1210; and human U937 and HL-60) with an IC_{50} for each cell line of 18, 20, 26, and 34 μM respectively (23). Fourth, normal cells are considered to be less sensitive to the effects of resveratrol. For example, the IC_{50} for resveratrol-induced apoptosis in the human leukemia cell lines THP1, HL60, U937, and WSU-CLL was 5.07, 15.72, 16.09, and 42.76 μM while the IC_{50} for normal human bone marrow cells was 60 μM (24).

The cardioprotective properties of resveratrol have been attributed to effects that include inhibition of platelet aggregation, vasorelaxation, improvements in serum cholesterol and triglycerides, inhibition of inflammation, reactive oxygen species scavenging, and reduced myocardial ischemic reperfusion injury. Vasorelaxation is mediated by the nitric oxide-cGMP pathway, and expression of nitric oxide synthase.

Resveratrol has been shown to significantly reduce proadhesion molecules such as soluble intracellular adhesion molecule-1 (sICAM-1), endothelial leukocyte adhesion molecule-1 (sE-Selectin), and vascular cell adhesion molecule-1 (sVCAM-1) in reperfusion models. In addition, resveratrol suppressed the aberrant expression of tissue factor and cytokines in vascular cells (3).

As mentioned above, resveratrol has antioxidant properties and is able to promote the activities of antioxidants such as glutathione and antioxidant enzymes such as superoxide dismutase and catalase (12, 25). Resveratrol concentrations of 1.25 μM, 2.5 μM, and 5μM were able to reduce the incidence of H_2O_2 (100 μM) induced DNA single strand breaks in murine leukemia cells (L1210), human myelogenous leukemia cells (K562), and human promyelocytic leukemia cells (HL-60) following a 24 hour pre-treatment with resveratrol (26). Inhibition of lipid peroxidation of cell membranes has also been shown in mouse macrophages (RAW 264.7) exposed to ·HO radicals generated by the Fenton reaction (27). Nitric oxide (NO) production by rat peritoneal macrophages was significantly inhibited in a dose dependent (10-100 μM) manner by resveratrol without affecting the viability of the cells (28).

Resveratrol has been shown to have anti-inflammatory properties. These effects are mediated by direct inhibitory action on COX-2 and suppression of COX-2 transcription upregulation in 184B5/HER cells (transformed human mammary epithelial cells) at resveratrol concentrations of 2.5-30 μM (29). In addition, resveratrol has been shown to inhibit both the cyclooxygenase and peroxidase activities of COX-1. COX-1 inhibition by resveratrol is time and concentration dependent with resveratrol binding to COX-1 occuring at a site different than arachidonic acid (30).

DNA replication, recombination, repair, relaxation, and telomere maintenance are all affected by resveratrol through physical and biochemical interactions. Resveratrol binds directly to DNA and RNA but does not act as a DNA intercalator. The ability of resveratrol to stabilize the DNA double-helical structure is thought to be the molecular basis for resveratrol's antimutagenic properties. An inhibitory effect on replicative DNA polymerase (pols) α and δ but not pols involved with DNA repair (β and λ) has been shown. Resveratrol has been classified as a topoisomerase class II inhibitor because it appears to interfere with enzymatic function without DNA strand break formation. Telomerase downregulation has also been demonstrated. Double strand break repair surveillance gene (*BRCA1, BRCA2, and TP53*) mRNA has been found to increase with resveratrol exposure in HBL100, MDA-MB-231, MCF-7, and MCF10A human breast carcinoma cells. In addition, resveratrol increases Rad51 protein, activates the ATM/ATR-Chk1/Chk2-Cdc25 pathway, and activates SIRT1 (31).

Cell-cycle distribution in a concentration and tissue-specific manner is modulated by resveratrol, resulting in suppression of cell cycle progression and cell cycle arrest at G0/G1 phase (human gastric adenocarcinoma cells – KATO-III, RF-1), G1 phase (human acute lymphoblastic leukemia cells – HSB-2 and neuroblastoma cells), G1/S transition (human epidermoid carcinoma cells – A431), S phase (human breast cancer cells – MCF-7, human lung carcinoma cells – A549, human promyelocytic leukemia cells – HL-60), S/G2 transition (human hepatoblastoma cells – HepG2, human colonic adenocarcinoma cells – Caco-2), and G2/M phase (human colon carcinoma cells – HT29) (32). Inhibition of expression of cyclin D1, D2, E and decreased expression of cdk 2, 4, 6 and induction of WAF1/p21 (human epidermoid carcinoma cells – A431) (33); and decreased

hyperphosphorylation of the retinoblastoma protein (human epidermoid carcinoma cells – A431) (34) have also been shown.

Resveratrol has been shown to induce apoptosis in cancer cells through caspase activation and upregulation of CD95-CD95L signaling (human promyelocytic leukemia cells – HL-60) (11); activation of p53-dependent transcription activity (mouse JB6 epidermal cells) (35, 36); activation of MAP kinases: ERK, JNKs, and p38 kinase (mouse JB6 epidermal cells) (36); decreased expression of the anti-apoptotic oncoprotein Bcl-2 (human promyelocytic leukemia cells HL-60) (37); and induction of Bax expression (rat azoxymethane-induced colon carcinogenesis) (38).

As implied with the preceding discussion, resveratrol has been investigated in a wide range of cancer cells lines and a small number of normal cell lines. These human cell lines include breast cancer (MCF-7, 184B5/HER, MDA-MB-231), ovarian cancer (Ovcar-3), liver cancer (HepG2), colon cancer (SW480), colorectal cancer (DLD1, HT29), pancreatic carcinoma (MiaPaCa2, S2-013, CD18), prostate carcinoma (LNCaP, PC-3, DU145, NB14), leukemia (HL-60), myeloid leukemia (U937), multiple myeloma (U266, RPMI 8226, KM3), T-cell acute lymphoblastic leukemia (MOLT-4), acute myeloid leukemia (OCIM2, OCI/AML3), and normal cells (PZ-HPV-7) (14, 38, 39). A number of mouse cell lines have also been used including epidermal cells (JB6), mastocytoma cells (P-815), lymphoblastic leukemia cells (L1210-R2), and leukemia (32Dp210) (38). *In-vivo* models have included multiple mouse strains (BALB/c, C57Bl/6J, Apc(Min+), athymic nude, and F344, SKH-1 investigating leukemia, breast cancer, lung cancer, liver cancer, prostate cancer, and gastric cancer (14, 38). Other animal models include rats, hamsters, rabbits, and fish (14).

Studies in mice have used a wide range of doses: 0.6 mg/kg to 1500 mg/kg and either oral or intraperitoneal injection administration (40-45). Concentrations of resveratrol used in research have often been compared to the amounts available in food sources like grapes or peanuts. This idea is important if food sources are intended to be the primary source of resveratrol. However, it is essential to recognize that the pharmacologic doses of resveratrol as a single agent used in most studies are not directly comparable to the physiological doses found in food for a number of reasons. Clearly there are advantages for using food sources therapeutically. It is commonly recognized from the results of epidemiological studies that a diet rich in fruit and vegetables confers protective effects against the development of cancer (46,47). As a result there has been increasing interest in the use of phytochemicals as cancer preventives or as a means to reduce the clinical signs of cancer. Cancer prevention has emerged as an important means of controlling malignancy because of the recognition that cancer preventive agents can substantially reduce the subsequent development of invasive cancer (48). Utilizing phytochemicals found in the diet offers a number of advantages for individuals at high risk for the development of neoplasia. These include 1) ease of use, 2) suitability for oral administration, 3) minimal toxicity, 4) suitability for prolonged used, 5) availability, and 6) simultaneous affect on multiple cellular pathways that are involved with carcinogenesis.

Phytochemicals "fit into a mechanism-based approach that targets whole pathways and sets of intracellular events rather than a single enzyme, as seen with many synthetic drugs" (49). This is important because it is becoming increasingly apparent that an approach that targets multiple cellular events is more likely to provide an effective

strategy for cancer therapy than a single event approach. Numerous phytochemicals have been investigated for their cancer preventive properties and it is clear that the process of carcinogenesis is affected in multiple ways. This includes acting as reactive oxygen species scavengers, modulation of inflammation, modulation of the immune response, cell cycle arrest, enhanced apoptosis of damaged cells, inhibition of angiogenesis, and modification of phase I and II enzymes (50). Modulation of specific molecular events has also been evaluated. Examples of studied proteins include p53, Rb/E2F, cyclins, cyclin-dependent kinases (CDKs), NF-κB, AP1, mitogen activated protein kinases MAPK), protein kinase C (PKC), phosphatyidylinositol 3-kinase (PI3K), inhibitors of apoptosis, COX, NOS, and sirtuin 1 (49,51).

While specific phytochemicals have been studied for their cancer preventive properties, it is clear that the plant matrix contains multiple phytochemicals and that many of these phytochemicals have individual and collective cancer preventive properties. Multiple phytochemicals within a plant can have similar cell and molecular targets and actions in mammalian cells (52-54). For example, it has been found that *trans*-resveratrol, *cis*-resveratrol, *trans*-piceid, *cis*-piceid, and quercetin, which are all found in *Polygonum cuspidatum*, all inhibit activation of NF-κB (55-58). *Trans*-piceatannol (*trans*-3,4,3',5'-tetrahydroxystilbene) and *trans*-pterostilbene (3,5-dimethoxy-4'-hydroxystilbene) have structures similar to *trans*-resveratrol and molecular effects similar to resveratrol (Figures 1 and 2). Piceatannol varies from resveratrol by only 1 hydroxyl group and pterostilbene has 2 methyl groups in place of the respective hydroxyl groups. These compounds have been shown to have properties similar to resveratrol. *Trans*-piceatannol has been shown to inhibit tyrosine kinase activity involved in cell

proliferation, tubulin phosphorylation, and phosphorylation of DNA transcription factors (59). Further, piceatannol was found to induce apoptosis in lymphoma cells BJAB through caspase-3 activation and was a more effective inducer of apoptosis in primary leukemic lymphoblasts than resveratrol in a concentration dependent manner (60). In the

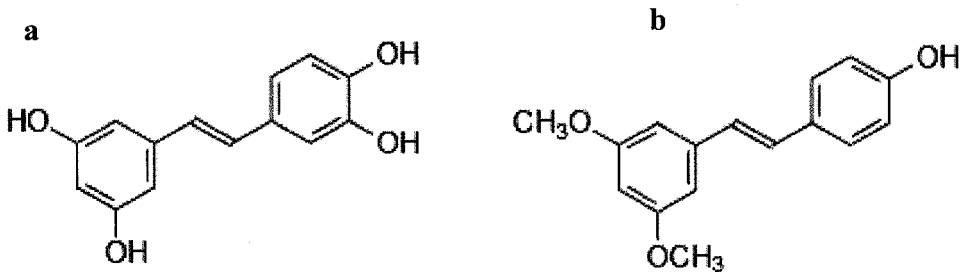

Figure 1. This figure demonstrates the structural similarities of *trans*-piceatannol (a) and *trans*-pterostilbene (b). The piceatannol varies from resveratrol 1 hydroxyl group and the pterostilbene by the replacement of 2 hydroxyl groups with methyl groups. Adapted from Signorelli *et al* (49).

leukemic cell line KBM-5, piceatannol inhibited TNF-mediated NF-κB activation in a concentration dependent fashion (61); induced apoptosis in the leukemia cell line U937 by down regulating Bcl-2 and activating caspase-3 (62); and induced apoptosis in leukemia cell lines HL60, Hl60R, K562, and HUT78 and increased cell accumulation in S phase in HL60 and K562 cells (63). In addition to piceatannol being present in the muscadine grape extract, resveratrol is metabolized to piceatannol by the cytochrome P450 enzymes CYP1B1 and CYP1A2 (59,64). *Trans*-ptersotilbene has also been shown to have similar properties as resveratrol, acting as an antioxidant (65); moderately inhibiting COX-1 and weakly inhibiting COX-2 (65); inducing apoptosis in leukemia cell lines (HL60, Hl60R, K562, and HUT78 (63); and cell cycle accumulation in S phase in HL60 and K562 cells (63).

It has also been shown that some phytochemicals must be altered prior to absorption in the intestinal tract. The glucosinolates found in cruciferous vegetables provide such an example. Glucosinolates have not been shown to have any anticarcinogenic activity. However, when acted on by the plant enzyme myrosinase, the glucosinolate is hydrolyzed to release the glucose. Myrosinase is compartmentalized in the plant and comes into contact with the glucosinolate when the plant is crushed or chopped. Hydrolysis rearrangement results in isothiocyanate or nitrile formation: glucobrassicin and progoitrin are hydrolyzed to indole-3-carbinol and crambene respectively. These glucosinolate hydrolysis products act as phase II enzyme inducers which are thought to protect cells from toxic or carcinogen induced damage.

Synergism is also known to occur between phytochemicals. This synergism results in increased levels of activity that are significantly greater than the activity observed by a single compound. For example, it has been shown that glucosinolate hydrolysis products (crambene and indole-3-carbinol) from broccoli when given to rats individually crambene (50 mg/kg) and indole-3-carbinol (56 mg/kg) daily for 7 days resulted in an increase in hepatic phase II enzymes quinone reductase and glutathione S-transferase subunit Ya2. When both of these compounds were given simultaneously there was an increase in both quinone reductase and glutathione S-transferase which was significantly greater than the additive effect of either compound alone (66). Subsequently it has been shown that crambene significantly activates the antioxidant response element (ARE) but not the xenobiotic response element (XRE) of the regulatory region of the quinine reductase gene. In addition, indole-3-carbinol acid condensates co-

activate the ARE and XRE resulting in a synergistic up regulation of quinone reductase when both crambene and indole-3-carbinol acid are present (67).

The number of newly diagnosed cancer cases is expected to double in the United States in the next 25-30 years (68). One method that has been advocated to address this problem has been the use of phytochemicals because phytochemicals affect an array of molecular pathways that can influence carcinogenesis, can be easy to administer, and are generally readily available. Using dietary substances as cancer preventives has the potential of being non-toxic, inexpensive, and can be taken orally (69). Cancer preventives can be used to prevent, slow progression, or delay carcinogenesis (48,50,70,71). Using biologic responses as a guide, the number of active compounds that have been identified has increased from 200 (72) to over 1,000 different phytochemicals that possess cancer preventive activities (51). Substances under investigation include retinoids, carotenoids, flavonoids, polyphenols, protease inhibitors, sulfides, terpens, n-3 polyunsaturated fatty acids, folic acid, vitamins C and E, and other antioxidants like glutathione (69,71-75). This approach is important because whole grains, fruits, and vegetables have a wide spectrum of phytochemicals that have the potential to modulate cancer development by modulating a variety of cellular pathways. It is important to note that in 2007 the World Cancer Research Fund and American Institute for Cancer Research released their Food, Nutrition, Physical Activity, and the Prevention of Cancer: a Global Perspective report. In this report, a panel of experts performed a "comprehensive evaluation of the relevant types of epidemiological and experimental evidence" to determine the effect of food on cancer risk. Based on the findings from cohort studies conducted in approximately the last 10 years, cancer protection from

vegetables and fruits are less impressive than previously reported and there was no evidence that was judged to be convincing (strongest evidence). However, a number of studies supported a judgment of probable protection. For lymphoid and hemopeoietic cancers, 1 cohort and 5 case-control studies showed a statistically significant association between increased vegetable and fruit consumption and cancer risk (76).

Resveratrol is found in the free form (aglycone) of *trans*-resveratrol, which is the most commonly used form in both *in vitro* and *in vivo* studies, and the *trans*-resveratrol glycoside (*trans*-piceid) and the *cis*-resveratrol aglycone and glycoside (Figure 2). The resveratrol glycosides generally predominate compared to the aglycone form in food because it is felt to protect the resveratrol from degradation in the plant. To give some perspective on concentrations of resveratrol in foods, *trans*-piceid, *cis*-piceid, *trans*-resveratrol, and *cis*-resveratrol were measured in 36 grape juices by Romero-Perez *et al*

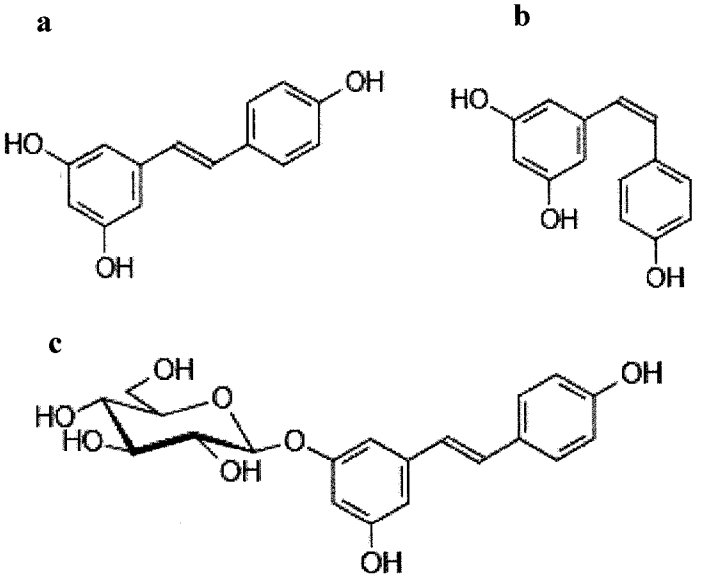

Figure 2. The basic structure of *trans*-resveratrol (a) aglycone, *cis*-resveratrol (b) aglycone, and *trans*-piceid (c) the resveratrol glycoside is shown. Adapted from Signorelli *et al* (49).

with the following mean results: red grape juice – *trans*-piceid 3.38 mg/L, *cis*-piceid 0.79 mg/L, *trans*-resveratrol 0.50 mg/L, and *cis*-piceid 0.06 mg/L and white grape juice – *trans*-piceid 0.18 mg/L, *cis*-piceid 0.26 mg/L, *trans*-resveratrol 0.05 mg/L, and *cis*-piceid not detected (77). A study investigating a large number (120) of Portuguese and French wines found the following mean results: red wine – *trans*-piceid 11.6 mg/L, *cis*-piceid 4.3 mg/L, *trans*-resveratrol 1.3 mg/L, and *cis*-piceid 2.5 mg/L and white wine – *trans*-piceid 3.0 mg/L, *cis*-piceid 1.4 mg/L, *trans*-resveratrol 0.6 mg/L, and *cis*-piceid 0.3 mg/L (78). In natural peanut butter, *trans*-resveratrol (0.534-0.753 μg/g, mean 0.652 μg/g n=7) and *trans*-piceid (0.073-0.225 μg/g, mean 0.143 μg/g n=7) were found (79); in a survey of commercial peanut products (including peanut butter), mean *trans*-resveratrol was found to range from 0.018 to 7.873 μg/g (n=28 different products) (80); and in a variety of peanut cultivars, mean *trans*-resveratrol ranged from 0.022 to 1.792 μg/g (n=15 cultivars) (81). Obviously, the resveratrol concentrations found in food make it difficult or impractical to deliver resveratrol in pharmacological doses using food if only the resveratrol content is considered to be active.

In addition to the preceding forms of resveratrol that were just discussed, foods like grapes are known to contain over 1600 constituents (82). Certain of these constituents may work together to promote beneficial effects even in the low concentrations present. With this is in mind, a number of authorities have postulated that low dose combination therapy strategies can be more effective for cancer prevention than those using individual agents (83, 84). Combination therapies have been shown to be more effective than single agent therapies for many clinical problems including cancer therapy and pain management. This combination therapy approach has been investigated

using the $APC^{Min/+}$ mouse, a model of human familial adenomatous polyposis. These mice were treated with a combination of sulindac (a nonspecific nonsteroidal anti-inflammatory) and EKI-569 (an irreversible inhibitor of the epidermal factor receptor kinase). A significant protection against intestinal tumorigenesis was found even though the sulindac dose used was ineffective as a single agent (85). A recent randomized placebo-controlled, double-blind trial investigating the recurrence of colorectal adenomas in human patients using low doses of difluoromethylorinithine (a polyamine synthesis inhibitor) and sulindac also demonstrated a significant decrease (70%) in recurrence in all adenomas (86).

When considering the translation of studies using the aglycone form of resveratrol to *in-vivo* research understanding resveratrol absorption, metabolism, and tissue distribution is essential. This need for pharmacokinetic information is highlighted by a study investigating prevention of benzo[a]pyrene induced lung cancer by resveratrol in which resveratrol, 6-8 mg/kg daily, was given for 7 months. The resveratrol did not reduce the incidence of lung tumors in these mice and did not significantly change CYP1A1 or CYP1B1 gene expression in any group (87) in spite of *in-vitro* data indicating that resveratrol did inhibit CYP1A1 and CYP1B1 gene expression and formation of DNA adducts in benzo[a]pyrene treated human bronchial epithelial cell lines (BEP2D and BEAS-2B) (88). However, the negative results in the mouse study are not surprising when it was determined that there were no detectible concentrations of conjugated or unconjugated resveratrol found in the lung tissue even though resveratrol was present in the intestines and feces (87). Understanding the tissue concentrations of

resveratrol would also greatly facilitate use of *in-vitro* resveratrol results when designing *in-vivo* studies.

In an early pharmacokinetic study, the cancer inhibition property of resveratrol was attributed to resveratrol's low bioavailability. This study erroneously considered the free form of resveratrol only (Figure 2) and not the conjugated forms (Figure 3) in tissues such as plasma, liver, kidney, and lung. Resveratrol at a dose of 20 mg/kg was detected within 2.5 minutes in the plasma after oral administration and declined steadily to minimal levels during the 60 minutes of the study (44). This early (within 15 minutes) detection of resveratrol in serum after oral administration was confirmed in another study. However, *trans*-resveratrol-3-sulfate and *trans*-resveratrol-3-O-glucuronide

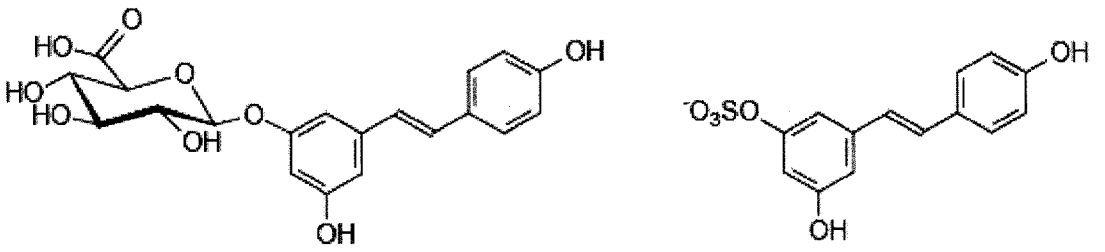

Figure 3. The structure of *trans*-resveratrol glucuronide and *trans*-resveratrol sulfate are shown. Adapted from Signorelli *et al* (49).

were also detected with the concentration of the resveratrol sulfate (13 μM) almost 3 times greater than the resveratrol glucuronide (5 μM) compared to the trace amounts of unconjugated resveratrol (89). Additional mouse studies have shown that *trans*-resveratrol glucuronide conjugate was detected in plasma at 5 minutes following oral administration (200 mg/kg as fed in the diet), peaked at 30 minutes, and was maintained for the 90 minutes of the study (41). This study also showed that resveratrol is absorbed

in the intestinal tract and undergoes glucuronidation before appearing in the blood. The presence of only trace amounts of unconjugated resveratrol was also confirmed. Both resveratrol sulfate and glucuronide forms were still present at 3 hours with the speculation that the conjugated resveratrol was distributed to the tissue and was slowly being cleared (89).

Using metabolic C^{14}-biolabelling of *trans*-resveratrol to study resveratrol tissue distribution following oral administration, Vitrac *et al.* sampled at 1.5, 3, and 6 hours after ingestion (90). At 1.5 hours radioactivity in the blood was low and did not increase significantly as would be expected from other studies investigating earlier post-administration times (41). Radioactivity in the urine and bile was high at 1.5 hours. At 3 hours the highest level of radioactivity was found in the duodenum, kidney, lung, liver, and spleen in descending order. The kidney was found to mainly contain the more polar glucuronides. Three hours after a 66 mg/kg dose of *trans*-resveratrol, 25 μM was found in the liver and 30 μM in the kidney (90).

Cellular accumulation of *trans*-resveratrol (150 μM) was found to be higher than trans-piceid (150 μM) in a cultured human intestinal cell (Caco-2) monolayer over a 2-30 minute period. While *trans*-resveratrol undergoes passive transport across the apical membrane, *trans*-piceid was actively transported via the sodium-dependent glucose cotransporter SGLT1 (91). Interestingly, *trans*-piceid can undergo hydrolysis in enterocytes through cleavage by cytosolic β-glucosidase after passing the brush border, or deglycosylation on the luminal intestinal surface by membrane bound lactase phlorizin hydrolase. The aglycone form of resveratrol can be acted on in the enterocyte to become conjugated as a glucuronide or a sulfate before release into the blood. This process of

deglycosylation, conjugation, and absorption is rapid with resveratrol glucuronide being detected in the blood within 5 minutes of oral administration (41). Efflux of the conjugated resveratrol is mediated by the multidrug resistance-associated protein 2 (MDRP2) (91). There is wide tissue distribution as already described with the liver having the highest level of conjugated resveratrol and metabolites and the kidney is the major route of elimination. Conjugated resveratrol has been detected in the tissue and blood for at least 6 hours post-administration indicating that it is possible to achieve prolonged tissue and systemic levels of conjugated resveratrol. In addition, it is important to note that significant levels of radioactivity were found in the spleen (90).

Ionizing radiation is known to create DNA damage that can lead to mutagenesis and carcinogenesis depending on the total dose, dose rate of delivery, and animal species (92-94). Radiation exposure can occur from both occupational and therapeutic sources. In addition, increasing survival following radiation therapy for cancer has led to increased risk of therapy-induced secondary cancers including acute myeloid leukemia (t-AML) (95,96). AML is a disease that can have a fulminating onset and can be rapidly fatal without appropriate therapy (97). Furthermore, t-AML carries a worse prognosis compared to de novo AML (98). Therefore, identification of cost effective, nontoxic methods to reduce the incidence of chromosome aberrations that potentially lead to t-AML is important. While a range of chromosome abnormalities have been identified in humans associated with AML, some authorities report increased incidence of PU.1 (*Sfpi1*) gene mutations in 7% of human AML patients (99) however, other studies have not confirmed this finding (100). Interestingly, even though the PU.1 gene has not

always undergone mutation, a decrease in PU.1 expression resulting from inactivity of other transcription factors has been reported associated with human AML (101-103).

The PU.1 protein is an ETS transcription factor that is restricted to blood cells. PU.1 is considered a transcriptional master regulator of myeloid and lymphoid cells which is evidenced by the presence of a PU.1-binding motif in the regulator sequences of almost all myeloid-specific and many lymphoid-specific genes. In addition, PU.1 is detectable in hematopoeitic stem cells (HSC), common myeloid progenitor cells (CMP), common lymphoid progenitor cells (CLP), and in mature myeloid cells. PU.1 is important for the HSC self repopulation and differentiation into CMP and CLP. While other transcription factors play a role in maturation and differentiation of leukocytes in the bone marrow, it is the response to graded levels of the PU.1 protein that is essential for differentiation of the granulocyte/monocyte progenitor cells (GMP) into granulocytes or monocytes (104). Increased PU.1 protein favors monocyte commitment of GMP cells (105).

The CBA mouse is an ideal model for studying radiation-induced AML. This mouse strain has a low incidence of background AML (<1%) and develops AML following irradiation. A single 3 Gy dose of ionizing radiation provided the maximum incidence of AML (25%) in CBA mice (106,107). A genetic instability has been identified on chromosome 2 in the D-E region in CBA mice with radiation-induced AML that includes the PU.1 gene (108,109). Interestingly, the development of AML in these mice results from a combination of the deletion of a single PU.1 gene allele and a missense mutation in the remaining PU.1 allele in the DNA-binding domain (109,110).

This missense mutation results in an inability of the PU.1 protein to bind DNA properly (109).

When considering resveratrol as a radioprotectant it is important to consider studies investigating the impact of exposing cells to resveratrol at the time of irradiation. Using K-562 (chronic myeloid leukemia) and IM-9 (multiple myeloma) cells and resveratrol concentrations of 0-200 μM and 0-8 Gy X-irradiation, resveratrol was shown to increase apoptosis following irradiation in a dose and cell line dependent manner (111). Cells were examined after 72 hours of resveratrol treatment but cell cycle distribution was not examined at the 24 and 48 hour end points. In a study using normal human lymphoblastoid cells (AHH-1), cells underwent a partial arrest in early S phase after 6 hours with continued accumulation in S phase with 24 hours of resveratrol (100 μM) exposure (112). S phase arrest was also demonstrated in human cervical carcinoma cell lines (HeLa and SiHa) and human non-small cell lung cancer cells (NCI-H838) as a component of a radiosensitizing effect of resveratrol (10-25 μM and 0-50 μM respectively) (113,114).

There are a number of important reasons why resveratrol has the potential to reduce radiation-induced chromosome damage and act as a preventive for radiation-induced cancer. First, resveratrol has the ability to induce cell cycle arrest in S phase. Second, prolonging S phase provides the opportunity for longer DNA repair time. This is important since resveratrol has been shown to positively influence DNA metabolism associated with DNA repair. Third, in those cells that are too damaged for effective repair, apoptosis is induced. The combination of these 3 processes could result in improved genome stability by either repairing the damage or eliminating unrepairable

cells. This study explored the potential for resveratrol to prevent events that lead to radiation-induced AML. Mice were divided into control groups that include 1) no treatment, 2) resveratrol only (100 mg/kg), 3) muscadine grape extract only (dosed at 5.73 μg/kg *trans*-resveratrol), and 4) radiation only (3 Gy γ-radiation) with end points at 1, 7, and 30 days post-irradiation. Experimental groups consisted of 1) resveratrol administered before radiation, 2) resveratrol initiated after radiation, 3) resveratrol before radiation with a range of doses, and 4) muscadine grape extract started before radiation using a range of doses. Metaphase bone marrow cells were scored for chromosome aberrations and loss of the PU.1 gene.

Chapter 2 has been published in *Radiation Research* in association with the indicated authors. Chapters 3-5 are formatted for submission for publication with the indicated authors. The final chapter (6) summarizes the findings of each group of experiments and brings the results together for discussion of the proposed mechanisms responsible for the reduction in radiation-induced chromosome aberrations and PU.1 gene loss. Future directions for research are also discussed.

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- Chapter 2 -

Resveratrol Reduces Radiation-induced Chromosome Aberration Frequencies in Mouse Bone Marrow Cells

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Abstract

Resveratrol, a polyphenol compound with reported antioxidant and anticarcinogenic effects, a wide range of molecular targets, and toxicity only at extreme doses, has received considerable attention. In this study we evaluated the radioprotective effect of orally administered resveratrol on the frequencies of chromosome aberrations in irradiated mouse bone marrow cells. CBA/CaJ mice were divided into 4 groups: 1) no treatment, 2) resveratrol only, 3) radiation only, and 4) resveratrol and radiation. Resveratrol treatment (100 mg/kg daily) was initiated 2 days prior to radiation. Bone marrow was then harvested at 1 and 30 days following a single 3 Gy dose of whole body γ-radiation. A statistically significant ($p<0.05$) reduction in the mean total chromosome aberration frequency per metaphase at both time points post-irradiation in the resveratrol and radiation group compared to the radiation only group was observed. This study is the first to show that resveratrol *in-vivo* has radioprotective effects. These results support use of resveratrol as a radioprotectant with the potential for widespread application.

Introduction

Ionizing radiation has been shown to induce DNA damage that can lead to mutagenesis and carcinogenesis depending on the total dose, dose rate of delivery, and animal species (1-3). Concern about radiation damage to normal tissues from occupational and therapeutic sources and the need for nontoxic protective compounds has prompted interest in the use of dietary compounds and medicinal plants for radiation protection (4,5). One dietary compound, resveratrol (*trans*-3,5,4'-trihydroxystilbene), is found in a wide variety of plant species including grapes, peanuts, blueberries, bilberries,

cranberries, lingonberries, and the weed *Polygonum cuspidatum* (6,7) and has received considerable attention for its reputed health benefits, including acting as a cardioprotectant and chemopreventive (8). Resveratrol's antioxidant effects, its ability to induce apoptosis and cell cycle arrest, and its low toxicity, make resveratrol an attractive candidate for radioprotection of normal cells and cancer prevention.

Resveratrol's antioxidant properties are mediated by its ability to scavenge free radicals and to promote the activities of antioxidants such as glutathione and antioxidant enzymes such as superoxide dismutase and catalase (9,10). Other studies have revealed resveratrol's ability to induce apoptosis in cancer cells via caspase activation and upregulation of CD95-CD95L signaling (11); to activate p53-dependent transcription activity (12); to activate MAP kinases: ERK, JNKs, and p38 kinase (13); to decrease expression of the anti-apoptotic oncoprotein Bcl-2 (14); and to induce Bax expression (15). Resveratrol also modulates cell-cycle distribution in a concentration and tissue-specific manner, resulting in suppression of cell cycle progression and cell cycle arrest at G0/G1 phase, G1 phase, G1/S transition, S phase, S/G2 transition, and G2/M phase (16). There is an inhibition of expression of cyclin D1, D2, E and decreased expression of cdk 2, 4, 6 (16,17); induction of WAF1/p21 (17); decreased hyperphosphorylation of retinoblastoma protein (17); and inhibition of ribonucelotide reductase and DNA polymerase activity (18). Interestingly, some studies have shown an increase in DNA synthesis (16) while 1 study reports a dose dependent biphasic effect on DNA synthesis (19). It has been speculated that slowing the cell cycle would benefit the cell by allowing increased time for repair; however, to our knowledge this has not been demonstrated using resveratrol.

A relatively nontoxic compound with multiple molecular targets, resveratrol has the potential to act as a radioprotectant in normal cells exposed to the damaging effects of ionizing radiation. The goal of the study reported here was to determine if resveratrol (100 mg/kg/day) initiated two days prior to 3-Gy whole body irradiation would result in a reduction in radiation-induced damage to bone marrow cells in mice.

Materials and Methods

Animals

Nine week old, male CBA/CaJ mice (24.7±0.7 g) obtained from Jackson Laboratory were used in this study. Mice were allowed to acclimate for approximately 1 week prior to use. The mice were housed and maintained according to the approved standards in the Laboratory Animal Resources facility at Colorado State University (CSU). During each phase of the study, mice were observed daily for general condition and activity levels. Any signs of illness or mortality were to be recorded and investigated. The Animal Care and Use Committee at CSU approved animal handling and experimental protocols.

Treatment groups

A total of 80, 10 week old, male CBA/CaJ mice were divided into two sets (1 day and 30 days) of 4 groups each, 10 mice each, as follows: 1) no treatment (No Tx), 2) resveratrol (RES) only (100 mg/kg), 3) radiation (RAD) only (3-Gy), and 4) RES+RAD (100 mg/kg and 3 Gy). One and 30 days following whole body irradiation, bone marrow from each mouse was individually harvested, processed, slides prepared and metaphase chromosome aberrations scored as described below.

Radiation

For radiation exposure, mice were placed as a group of 5 into a well ventilated plexiglass container. A total dose of 3 Gy γ-radiation, at a dose rate of 1.18 Gy/min, was delivered using a ^{137}Cs irradiator (J.L. Shepherd model 81-14).

Resveratrol Dose and Administration

Resveratrol (3,4',5-trihydroxy-*trans*-stilbene - Sigma-Aldrich) was dissolved in 100% ethanol to a concentration of 50 mg/ml. Prior to gavage, the resveratrol/ethanol dose was diluted as follows: 0.05 ml of resveratrol (50 mg/ml) in 0.2 ml distilled water for a final concentration of 10 mg/ml. Individual mice in each designated group received a resveratrol dose of 100 mg/kg administered by gavage every 24 hours for 2 days prior to irradiation, and again on the day of irradiation, 30 minutes prior to radiation exposure. For mice in the 30 day group, the resveratrol/ethanol mixture was added to the drinking water at a rate of 50 mg resveratrol per 100 ml, beginning on the morning following irradiation and continuing until the final day of the study period. The resveratrol stock and resveratrol water combination were protected from light by covering the container with aluminum foil. The resveratrol water combination was regularly changed.

Bone Marrow Collection

Colchicine (0.1 ml of a 0.5% solution) was administered by intraperitoneal (IP) injection to each mouse one hour prior to bone marrow collection. Bone marrow was flushed from each femur, tibia, and radius with PBS. The PBS bone marrow mixture was centrifuged at 1000 rpm for 5 min at 4°C. The cell concentrate was then placed into an incubation mixture (9 ml 0.075 M KCl, 1 ml EDTA trypsin, 200 μl colcemide), mixed, and incubated for 30 min at 37°C. Following incubation, 5 ml fresh fixative (3:1

methanol:acetic acid) was added, mixed, and centrifuged at 1000 rpm for 5 min. Cells were washed with fresh fixative 2 more times. The supernatant was decanted, a small amount of fixative added, and cells were dropped onto clean, wet microscope slides and allowed to dry.

Chromosome Aberration Scoring

Slides with bone marrow metaphase cells were stained with 10% Giemsa (Sigma-Aldrich) for 7 min, rinsed, air dried, and mounted with a glass coverslip. The slides were coded, blinded, and then 25 metaphases with 38-40 chromosomes each were scored for aberrations. Chromosome aberrations observed in bone marrow metaphases were scored as follows: 1) fragments (chromosome and chromatid types), identified as un-rejoined acentric fragments derived from a chromosome or chromatid severance, including terminal deletions and interstitial deletions not associated with an exchange process, 2) gaps, identified as an achromatic site along the length of a chromatid (or isochromatid) that was less than the width of the chromatid, 3) dicentrics, identified as a chromosome (or chromatid) end joined to another chromosome (or chromatid), and 4) Robertsonian translocations, identified as two chromosomes joined at their centromeres. All bone marrow metaphases were scored in the same fashion to allow consistent comparisons. Figure 1 shows representative photomicrographs from typical metaphase bone marrow cells from each treatment group on day 1 post-irradiation. After scoring, the slides were unblinded and results were compiled according to the respective treatment group. As a result of the quality of the morphology of the metaphase cells derived directly from bone marrow, categories of aberrations were combined and tallied to give a total that was used for statistical calculations.

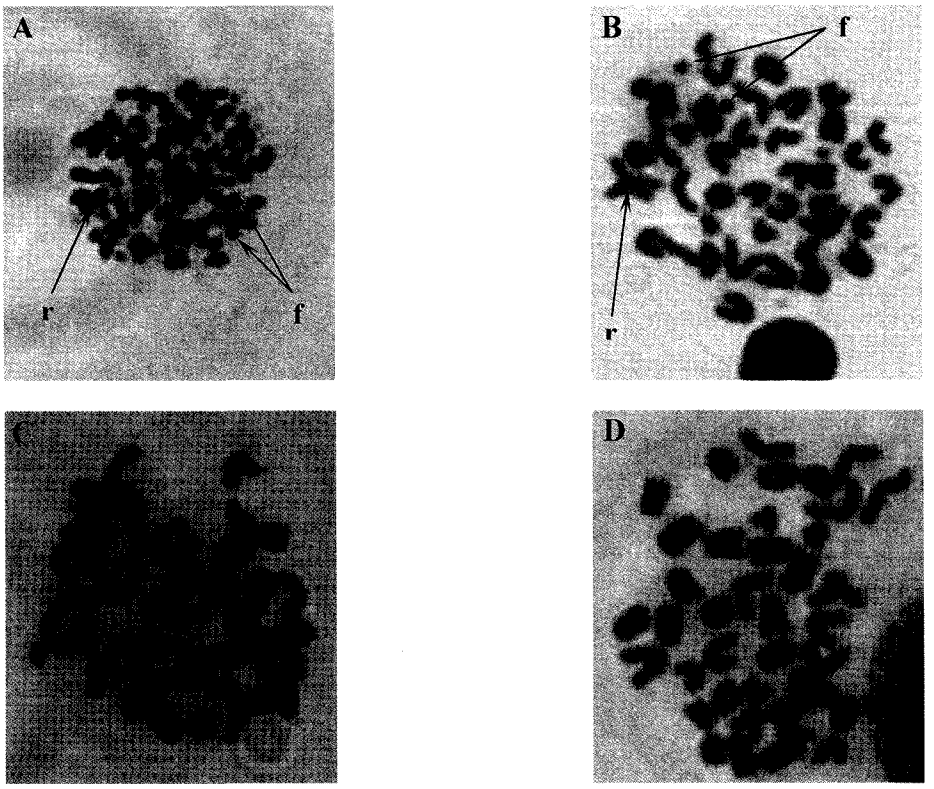

Figure 1. Photomicrographs of metaphase bone marrow cells from each treatment group corresponding to 1 day post-irradiation: A – radiation only, B – resveratrol + radiation, C – no treatment, and D – resveratrol only. Arrows indicate chromosome fragments (f) and Robertsonian translocations (r).

Statistical Analysis

For statistical analysis, the following categories were compiled 1) fragments: chromosome and chromatid types combined, 2) gaps: chromatid and isochromatid types combined, 3) dicentrics, 4) Robertsonian translocations, and 5) total chromosome aberrations. Statistical analysis consisted of evaluation of the mean total chromosome aberration frequency per time point for each treatment group. Total aberrations represent the sum of all chromosome aberration types observed. The data are presented as the mean total $\pm$ standard deviation of chromosome aberrations per bone marrow metaphase. Since

the data exhibited non-normality and heteroscedasticity, the non-parametric Kruskal-Wallis ANOVA was used for overall comparisons between the four treatment groups at the 2 time points, and Wilcoxon rank-sum tests were used to compare total chromosome aberration frequencies between pairs of groups.

Results

Fragments were the most common aberration seen in the RAD and RES+RAD groups followed by gaps, dicentrics, and Robertsonian translocations (Table 1). All chromosome aberration frequencies were found to vary by treatment group and time point (Table 1 and Figures 2 and 3). For fragments and total mean chromosome aberration frequencies, the Kruskal-Wallis ANOVA p-value was less than 0.0001 at days 1 and 30, while for gaps and dicentrics the p-value was less than 0.001 at day 1 but not at day 30 (Table 2). A low mean level of total background chromosome aberrations was observed in the bone marrow metaphase cells scored at days 1 and 30 for the No Tx group. This low background level was true for fragments, gaps, dicentrics, and Robertsonian translocations. In the RES group, a lower mean total of background chromosome aberrations at day 1 and 30 was noted compared to the No Tx group. Again, a similar relationship was observed for fragments, gaps, dicentrics, and Robertsonian translocations. However, the difference between the No Tx and RES groups did not rise to the level of statistical significance for any aberration category. The RAD group had the highest mean level of total chromosome aberrations, fragments, gaps, dicentrics, and Robertsonian translocations at days 1 and 30 following radiation exposure. On day 1, the RES+RAD group had the second highest mean level of total

TABLE 1

Mean Chromosome Aberrations per Bone Marrow Metaphase Cell Observed in Each Mouse Group at Each Time

	Total	Fragments	Gaps	Dicentrics	Robertsonian
No treatment					
Day 1	0.20±0.55	0.06±0.28	0.07±0.38	0.01±0.11	0.06±0.25
Day 30	0.14±0.45	0.02±0.15	0.04±0.27	0.01±0.09	0.07±0.25
Resveratrol					
Day 1	0.12±0.39	0.02±0.18	0.04±0.26	0.02±0.13	0.03±0.18
Day 30	0.07±0.27	0.01±0.13	0.02±0.15	0.00±0.06	0.03±0.18
Radiation					
Day 1	2.90±3.04	2.12±2.65	0.33±0.70	0.28±0.28	0.17±0.50
Day 30	0.51±0.87	0.22±0.61	0.13±0.55	0.01±0.11	0.14±0.39
Resveratrol + radiation					
Day 1	1.40±1.92	0.88±1.67	0.22±0.60	0.19±0.49	0.12±0.34
Day 30	0.18+0.47	0.02±0.14	0.06±0.30	0.00±0.06	0.09±0.31

Notes. Values are means ± SD based on 250 cells counted. Total includes fragments, gaps, dicentrics, and Robertsonian translocations. Fragments include chromosome and chromatid types, including interstitial deletions, terminal deletions, and breaks. Gaps include chromatid and isochromatid types. Dicentrics were of the chromatid type and Robertsonian translocations were of the chromosome type.

chromosome aberrations, fragments, gaps, dicentrics, and Robertsonian translocations; however, on day 30 the mean level of total chromosome aberrations fragments, gaps, dicentrics, and Robertsonian translocations for the RES+RAD group, were similar to the mean found for the No Tx group (Figures 2 and 3).

Statistically significant differences ($p<0.05$) were found on day 1 post-irradiation when comparing the means for total chromosome aberrations, fragments, gaps, dicentrics, and Robertsonian translocation frequencies between the No Tx vs. RAD groups, RES+RAD vs. RES groups, and RES+RAD vs. No Tx groups; but, only the mean

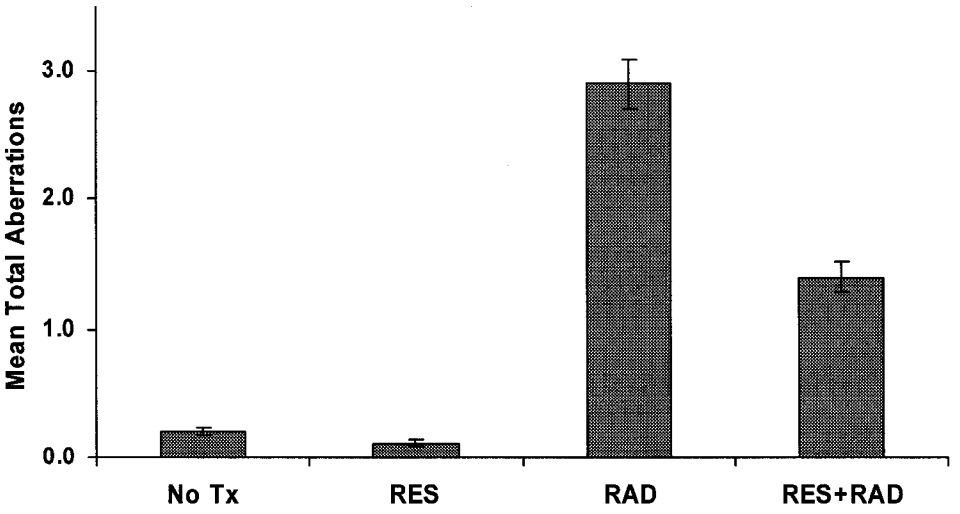

Figure 2. Total mean chromosome aberrations ± SE per bone marrow metaphase cell of each mouse group are shown for day 1 following 3 Gy whole-body irradiation. Note statistical significance at the $p<0.05$ level was found when comparing the no treatment (No Tx) group vs. the radiation (RAD) group and radiation (RAD) vs. resveratrol + radiation (RES+RAD) for mean total aberrations.

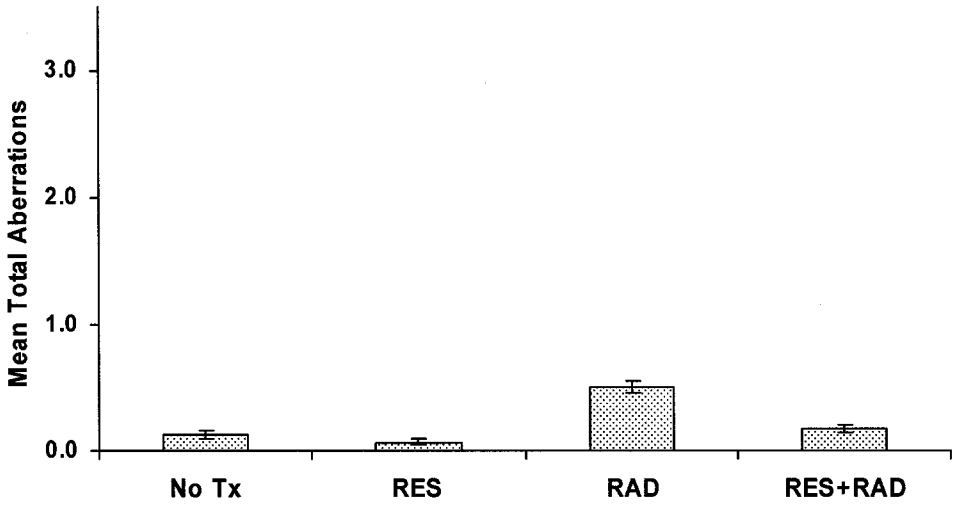

Figure 3. Total mean chromosome aberrations ± SE per bone marrow metaphase cell for each mouse group at 30 days post-irradiation are shown. Statistical significance at the $p<0.05$ level was found when comparing the no treatment (No Tx) group vs. the radiation (RAD) group and the radiation (RAD) vs. the resveratrol + radiation (RES+RAD) for mean total aberrations.

frequencies for total, fragments, and gaps when comparing RES+RAD vs. RAD groups (Table 2). On day 30 statistically significant differences ($p<0.05$) were found when comparing the mean for total chromosome aberrations for RES+RAD vs. RAD, RAD vs. No Tx groups, and RES+RAD vs. RES (Table 2). A statistically significant difference was not found for any chromosome aberration category (total, fragments, gaps, dicentrics, or Robertsonian translocations) when comparing RES+RAD vs. No Tx groups at day 30.

TABLE 2
Statistical Comparisons between Mouse Groups and Aberration Categories at Each Time

			Wilcoxon rank-sum tests ($p<0.05$)			
Event	Day	Kruskal-Wallis ANOVA (p-value) Overall	Resveratrol+ radiation vs radiation	Radiation vs no treatment	Resveratrol + radiation vs resveratrol	Resveratrol + radiation vs no treatment
Total	1	<0.0001	√	√	√	√
	30	<0.0001	√	√	√	ns
Fragments	1	<0.0001	√	√	√	√
	30	<0.0001	√	√	ns	ns
Gaps	1	<0.0001	√	√	√	√
	30	0.0476	ns	√	ns	ns
Dicentrics	1	<0.0001	ns	√	√	√
	30	0.6637	ns	ns	ns	ns
Robertsonian	1	<0.0001	ns	√	√	√
	30	0.0014	ns	√	√	ns

Note. The √ indicates a statistically significant relationship while the ns indicates that a significant relationship was not found at $p<0.05$.

Discussion

Resveratrol has been shown to sensitize cancer cells to the radiosensitizing effects of radiation *in-vitro* (20-22). However, only 1 study reported on the combination of resveratrol and ionizing radiation on normal cells (23). Unfortunately, this study did not clearly document if resveratrol combined with a single 5-Gy X-ray sensitized these

normal cells to radiation-induced DNA damage. Currently, there are no reports concerning the effects of combining resveratrol and radiation *in-vivo*. Our results indicate that resveratrol (100 mg/kg daily) administered as a bolus each day for 2 days and then 30 min prior to 3-Gy whole body irradiation on the third day, and then daily in the drinking water until the end of the study period, reduces the mean total chromosome aberration frequency observed in bone marrow metaphases at 1 and 30 days post-irradiation. The mean number of total chromosome aberrations per metaphase was reduced 2.1-fold at one day and 2.8-fold at 30 days in the RES+RAD group compared to the RAD only group. At 30 days post-radiation, the mean total aberrations were not statistically different between the RES+RAD group vs. No Tx group indicating that resveratrol is effective at reducing the mean of total chromosome aberrations to nearly normal background levels within 30 days (Figure 3). The difference in the mean total chromosome aberrations between the No Tx vs. RES groups was not statistically significant indicating that resveratrol did not induce observable chromosome aberrations at the 100 mg/kg oral dose. This lack of cytogenetic toxicity is consistent with the effect that resveratrol has on apoptosis and cell viability in normal human peripheral blood mononuclear cells and normal mouse bone marrow cells (24,25).

A review of the results of the experiments reported here provides 3 basic observations. First, radiation-induced chromosome aberrations (total, fragments, gaps, dicentrics, and Robertsonian translocations) were significantly increased compared to mice not receiving radiation at day 1. This was also true except for dicentrics on day 30 where the frequency was similar to background levels. Second, resveratrol significantly reduces radiation-induced total, fragments, and gaps on day 1 compared to the irradiated

mice but not dicentrics or Robertsonian translocations. At 30 days resveratrol significantly reduced the radiation-induced total and fragments but not gaps, dicentrics, or Robertsonian translocations. Third, resveratrol was able to reduce radiation-induced total, fragments, gaps, and Robertsonian translocations to background levels ($p>0.05$) at 30 days.

While the specific mechanism responsible for the observed effect of resveratrol reducing the frequency of chromosome aberrations was not evaluated in this study, there are a number of possible mechanism(s) described in published resveratrol studies. Since γ-radiation is known to induce cellular damage in part though oxidative processes, it is possible that the significant reduction in observed chromosomal damage observed in the RES+RAD group compared to the RAD group is the result of the direct antioxidant properties of resveratrol or indirectly through induction of antioxidants like glutathione and increases in enzymes like superoxide dismutase and catalase (9,10). The ability of resveratrol to induce cell cycle arrest in S phase and/or at the G2/M transition, at concentrations of 15-20 μM, 30 μM, and 100 μM in leukemia cells (26, 27) and potentially allow a longer period of time for chromosomal repair following a DNA damaging event could contribute to reduction in the frequency of chromosome aberrations seen in metaphase spreads in the RES+RAD group. Induction of apoptosis in damaged or abnormal cells at concentrations of 10-200 μM (11,14,28) would also facilitate a reduction in the observed frequency of chromosome aberrations in the RES+RAD group. It is likely that all these properties of resveratrol contribute to the observed reduction in chromosome aberrations observed at each time point in this study.

The resveratrol dose selected here was expected to produce tissue concentrations consistent with those concentrations (10-50 μM) that have been shown to have effects *in-vitro* on molecular targets. Pharmacokinetic studies in mice have used a wide range of doses: 0.6 mg/kg to 1500 mg/kg and either oral or IP administration (29-32). One study using an oral dose of 50 mg/kg achieved a liver and kidney concentration of 25-30 μM and resveratrol conjugates were still present at the final 6 hour time point (33).

A number of resveratrol toxicity studies using extremely large doses (2000-4000 mg/kg mice and 3000 mg/kg rats) have shown renal toxicity (34,35), while a daily 20 mg/kg dose in rats did not (36). In the study reported here, we found that resveratrol at a daily dose of 100 mg/kg did not induce chromosome aberrations after 30 days of ingestion.

Bone marrow is a complex grouping of different cell types including leukocytes, erythrocytes, megakaryocytes, and stromal cells at different stages of maturation. The exact kinetics of each of these bone marrow cells in the mouse is unclear. However, studies have evaluated bone marrow cells and bone marrow stromal cells following irradiation (37-40). These studies show that radiation-induced aberrations in bone marrow-derived cell populations can persist for months after radiation (38) and that bone marrow stromal cells continue to show radiation-induced alterations in the bone marrow stromal compartment *in-vitro* 6 months after 2-Gy irradiation (40). Bone marrow stromal cells have reduced capacity for self-renewal and altered blocking of apoptosis in attached hematopoietic stem cells following irradiation (39). These factors likely contribute to the continued presence of bone marrow chromosome aberrations 30 days after whole body irradiation. With its wide range of cellular targets, it is possible that resveratrol has

beneficial effects not only on bone marrow hematopietic cells, but also on the stromal compartment.

In this study we determined that administration of resveratrol at a dose of 100 mg/kg daily, initiated 2 days prior to 3-Gy whole body irradiation, resulted in a statistically significant reduction in the mean total chromosome aberration frequency in mouse bone marrow cells. This reduction in chromosome aberration frequency is thought to be the result of a combination of resveratrol's previously identified cellular and molecular properties including its antioxidant activities and its ability to induce apoptosis and cell cycle arrest. Future studies will investigate these possibilities using this *in-vivo* model in an attempt to determine the specific contribution from each mechanism and the potential for resveratrol to exert its radioprotective and cancer preventive properties to reduce or prevent radiation-induced acute myeloid leukemia in at risk populations.

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- Chapter 3 -

Resveratrol Initiated Post-irradiation Reduces Radiation-induced Chromosome Aberration Frequencies in Mouse Bone Marrow Cells

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Abstract

Resveratrol has been shown to possess antioxidant properties, induce production of antioxidant substances, has anticarcinogenic effects, influences a wide range of molecular targets, and has toxicity only at extreme doses. Previously we demonstrated that resveratrol administered before radiation exposure reduces the observed frequency of chromosome aberrations in mouse bone marrow cells. In the current study, CBA/CaJ mice were divided into four groups: 1) no treatment, 2) resveratrol only, 3) radiation only, and 4) radiation and resveratrol to evaluate the effect of oral resveratrol initiated after irradiation on the frequency of chromosome aberrations in irradiated mouse bone marrow metaphase cells. Resveratrol at 100 mg/kg daily was initiated 2 hours or 2 days after radiation, continued daily for the designated time or given as a single dose, and bone marrow was harvested at 1, 7, or 30 days following a single 3 Gy dose of whole body γ-radiation. In addition, the frequency of micronuclei was determined in each group at day 1 post-irradiation. A statistically significant ($p<0.05$) reduction in the mean total chromosome aberration frequency per metaphase at all time points post-irradiation in the radiation plus resveratrol group compared to the radiation only group was observed. Further, there was not a significant ($p=0.5746$) increase in micronuclei in the radiation only compared to the radiation plus resveratrol group. These results demonstrate that resveratrol started after irradiation significantly reduces the frequency of chromosome aberrations in bone marrow cells.

Introduction

Ionizing radiation is known to create DNA damage that can lead to mutagenesis and carcinogenesis depending on the total dose, dose rate of delivery, and animal species (1-3). There is a growing interest in the study and use of dietary compounds, like resveratrol (*trans*-3,5,4'-trihydroxystilbene), for disease management including use for radioprotection (4-6). Resveratrol has been found in a wide range of plants including grapes, peanuts, blueberries, bilberries, cranberries, lingonberries, and the weed *Polygonum cuspidatum* (7-11). A number of resveratrol properties including, its antioxidant effects, its ability to induce apoptosis and cell cycle arrest, and its low toxicity, make resveratrol an attractive candidate for radioprotection and chemoprevention.

Antioxidant properties of resveratrol are seen in its ability to scavenge free radicals and to promote the activities of antioxidants such as glutathione and antioxidant enzymes such as superoxide dismutase and catalase (rat aortic smooth muscle cells and normal peripheral blood mononuclear cells) (12,13). In addition, resveratrol has been shown to induce apoptosis in cancer cells through caspase activation and upregulation of CD95-CD95L signaling (human promyelocytic leukemia cells – HL-60) (14); activation of p53-dependent transcription activity (mouse JB6 epidermal cells) (15,16); activation of MAP kinases: ERK, JNKs, and p38 kinase (mouse JB6 epidermal cells) (16); decreased expression of the anti-apoptotic oncoprotein Bcl-2 (human promyelocytic leukemia cells HL-60) (17); and induction of Bax expression (rat azoxymethane-induced colon carcinogenesis) (18). Modulation of cell-cycle distribution in a concentration and tissue-specific manner has also been identified, resulting in suppression of cell cycle

progression and cell cycle arrest at G0/G1 phase (human gastric adenocarcinoma cells – KATO-III, RF-1), G1 phase (human acute lymphoblastic leukemia cells – HSB-2 and neuroblastoma cells), G1/S transition (human epidermoid carcinoma cells – A431), S phase (human breast cancer cells – MCF-7, human lung carcinoma cells – A549, human promyelocytic leukemia cells – HL-60), S/G2 transition (human hepatoblastoma cells – HepG2, human colonic adenocarcinoma cells – Caco-2), and G2/M phase (human colon carcinoma cells – HT29) (19). Inhibition of expression of cyclin D1, D2, E and decreased expression of cdk 2, 4, 6 and induction of WAF1/p21 (human epidermoid carcinoma cells – A431) (20); and decreased hyperphosphorylation of the retinoblastoma protein (human epidermoid carcinoma cells – A431) (21) have also been shown. In addition, resveratrol can inhibit enzymes like ribonucleotide reductase (human breast cancer cells – MCF-7 and MDA-MB-231) (22), human DNA polymerases α and δ (23), human topoisomerase II (24), and telomerase (human breast cancer cells MCF-7) (25). Resveratrol also modulates DNA double-strand break repair in lymphoblastoid cell lines (TK6 and WTK1) independent of its effect on cell cycle arrest and apoptosis (26).

Resveratrol has been shown to be relatively nontoxic but was assessed here using the cytokinesis-block micronucleus assay. The cytokinesis-block micronucleus assay has been used to assess chromosome damage including chromosome loss and breakage resulting from genotoxic compounds or radiation. This method has the advantage that micronuclei can only be observed in cells that complete nuclear division. These cells have a binucleate appearance because cytokinesis is blocked by cytochalasin B (27).

Resveratrol has the potential to act as a radioprotectant in a concentration dependent manner in normal cells exposed to damaging levels of ionizing radiation. The

goal of the study reported here was to determine if resveratrol (100 mg/kg/day) initiated a) as a single dose at 2 hours, b) at 2 hours and continued, or c) at 2 days and continued following a single dose of 3 Gy whole body γ-radiation would result in a reduction in radiation-induced chromosome aberrations in mouse metaphase bone marrow cells at 1, 7, and 30 days post-irradiation.

Materials and Methods

Animals

Male CBA/CaJ mice, 8-9 weeks old, were obtained from Jackson Laboratory and acclimated for approximately 1 week prior to use. The mice were housed and maintained according to the approved standards in the Laboratory Animal Resources facility at Colorado State University (CSU). Activity and physical condition of the mice were observed daily and any abnormalities were recorded, reported, and investigated. The Animal Care and Use Committee at CSU approved animal handling and experimental protocols.

Treatment Groups

A total of 150, 9-10 week old, male CBA/CaJ mice (24.7±0.7 g) were divided into 3 experiment sets (1, 7, and 30 days) of 4-7 groups with 10 mice each for the cytogenetic study, as follows: 1) no treatment (No Tx), 2) resveratrol (RES) only (100 mg/kg), 3) radiation (RAD) only (3 Gy), and 4) radiation followed by resveratrol (RAD>Res) (3 Gy and 100 mg/kg). The RAD>Res group was divided into 3 treatment groups with bone marrow harvested at the indicated time points: 1) resveratrol administered by gavage 2 hours after irradiation as a single dose (RAD>Res 2hrs) with bone marrow harvested at 1,

7, and 30 days, 2) resveratrol administered by gavage 2 hours after irradiation and continued mixed in the drinking water (RAD>Res 2 hrs+) with bone marrow harvested at 30 days, and 3) resveratrol added to drinking water to deliver 100 mg/kg daily beginning 2 days after irradiation (RAD>Res 2 days+) with bone marrow harvested at 7 and 30 days. One, 7, and 30 days following whole body irradiation, bone marrow from each mouse was individually harvested, processed, placed on slides, and metaphase chromosome aberrations scored as described below.

For the cytokinesis-block micronuclei study, 9 week old, male CBA/CaJ mice were divided into 5 groups: 1) No Tx, 2) RES, 3) RAD, 4) resveratrol initiated before radiation (Res+RAD) (100 mg/kg and 3 Gy), and 5) RAD>Res (3 Gy and 100 mg/kg). In the RES and Res+RAD groups resveratrol was administered every 24 hours by gavage for 2 days and on the third day, 30 minutes before irradiation. For the RAD>Res group, resveratrol was started 2 hours after irradiation. In all groups, bone marrow was collected and placed in RPMI 1640 (Thermo Scientific) media containing 1 μg cytochalasin B (Fermentek) per 1 ml at a time corresponding to 24 hours after irradiation. Following 18 hours of incubation, cells were placed on slides, blinded, and scored for micronuclei as described below.

Radiation

Mice were placed as a group of 3-5 into a well-ventilated plexiglass container. γ-radiation was delivered using a ^{137}Cs irradiator – J.L. Shepherd model 81-14. Radiation was delivered to each mouse at a dose rate of 1.13 Gy/min to a total dose of 3 Gy based on dosimetry.

Resveratrol Dose and Administration

Prior to use, resveratrol (*trans*-resveratrol – Sigma-Aldrich) was dissolved in 100% ethanol to a concentration of 50 mg/ml. Just before administration, the resveratrol/ethanol dose was diluted in distilled water as follows for gavage: 0.05 ml of resveratrol (50 mg/ml) mixed with 0.2 ml distilled water. For the 7 and 30 day studies, the resveratrol/ethanol mixture was added to the drinking water at a rate of 50 mg of resveratrol per 100 ml of drinking water. At all times the resveratrol was protected from light by covering the container with aluminum foil. The resveratrol and drinking water mixture was regularly changed. Mice were divided into 3 treatment groups (cytogenetic study) and 4 treatment groups (micronuclei study) with defined end points as described in the *Treatment Groups* section above.

Bone Marrow Collection

Mice in the cytogenetic studies were administered colchicine by intraperitoneal (IP) injection (0.1 ml of 0.5%) 1 hour before bone marrow collection. For both the cytogenetic and micronuclei studies, bone marrow was obtained from each femur, tibia, and radius by flushing with PBS. This PBS and bone marrow mixture was centrifuged at 1000 rpm for 5 min at 4°C. Bone marrow cells intended for the micronuclei study were mixed with RPMI 1640 media containing 1 μg cytochalasin B per ml of media and incubated at 37°C for 18 hours. Following incubation (micronuclei assay) or bone marrow harvest (cytogenetic studies), the bone marrow cells were then mixed with an incubation mixture (9 ml 0.075 M KCl, 1 ml EDTA trypsin, 200 μl colcemid) and incubated for 30 min at 37°C. After incubation, 5 ml of fresh fixative (3:1 methanol:acetic acid) was added, mixed, and centrifuged at 1000 rpm for 5 min. Cells

were washed with fixative 2 more times. The supernatant was decanted and a small amount of fixative was added. Cells were dropped onto cleaned microscope slides and allowed to air dry.

Chromosome Aberration Scoring

Slides containing mouse bone marrow metaphase cells were stained with 10% Giemsa (Sigma-Aldrich) for 7 min, rinsed, air dried, and mounted with a glass coverslip. These slides were coded and blinded. Twenty-five metaphase cells with 38-40 chromosomes each were scored for aberrations. Chromosome aberrations observed in bone marrow metaphases were scored as follows: 1) fragments (chromosome and chromatid types) – identified as un-rejoined acentric fragments derived from a chromosome or chromatid severance, including terminal deletions and interstitial deletions not associated with an exchange process, 2) gaps – identified as an achromatic site along the length of a chromatid (or isochromatid) that was less than the width of the chromatid, 3) dicentrics – identified as a chromosome (or chromatid) end joined to another chromosome (or chromatid), and 4) Robertsonian translocations – identified as 2 chromosomes joined at their centromeres. All bone marrow metaphases were consistently scored for all groups to allow comparisons. Following scoring, the slides were unblinded and results were compiled according to the respective treatment group and end point. Photomicrographs of representative metaphase bone marrow cells from each treatment group are shown in Figure 1.

Cytokinesis-blocked Micronuclei Scoring

Slides containing mouse bone marrow cells previously treated with cytochalasin B were dried and stained with DAPI stain (Vector Laboratories) and mounted with a

glass coverslip. These slides were coded and blinded prior to scoring. Binucleate cells were evaluated for the presence of a nuclear fragment in close proximity. For each mouse, a total of 200-500 binucleate cells were examined and scored as positive or negative for presence of micronuclei.

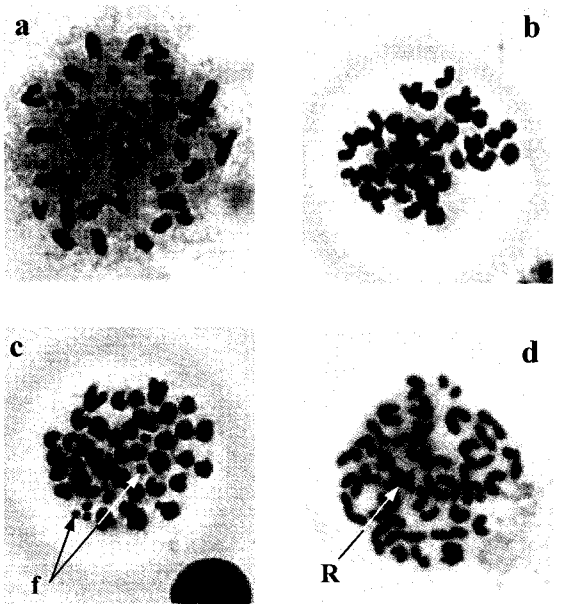

Figure 1. Photomicrographs showing representative bone marrow metaphase cells from mice in the a) no treatment, b) resveratrol only, c) radiation only at day 1 post-irradiation, and d) radiation 2 hours before resveratrol groups at day 1 post-irradiation. Arrows indicate chromosome aberrations: f - indicates acentric fragments derived from chromosome or chromatid severance and R - indicates a Robertsonian translocation.

Statistical Analysis

The following categories were compiled for statistical analysis: 1) fragments: chromosome and chromatid types combined, 2) gaps: chromatid and isochromatid types combined, 3) dicentrics, 4) Robertsonian translocations, and 5) total chromosome aberrations. Total aberrations represent the sum of all chromosome aberration types observed. Statistical analysis consisted of evaluation of each category for each time point and treatment group. The data are presented as the mean $\pm$ standard deviation of the total

and each chromosome aberration frequency type per bone marrow metaphase. Since the data exhibited non-normality and heteroscedasticity, non-parametric Wilcoxon rank-sum tests were used to compare chromosome aberration frequencies between pairs of groups.

The micronuclei results for each treatment group were used to calculate the mean ± standard deviation. Additionally, the Chi-square test was used to calculate p-values.

Results

Results of the cytogenetic analysis for the following control groups have been previously reported for days 1 and 30: no treatment, resveratrol only, and radiation only (6). Those results are summarized again here for clarity and ease of discussion. However, the day 7 control group results for the resveratrol and radiation groups are reported here for the first time (Table 1). Background levels of chromosome aberrations in the bone marrow metaphase cells scored at days 1, 7, and 30 for the no treatment and resveratrol groups were low (Figure 2). The differences between the no treatment and resveratrol only groups did not rise to the level of statistical significance for any aberration category. All chromosome aberrations (total, fragments, gaps, dicentrics, and Robertsonian translocations) were significantly increased at the $p<0.05$ level on day 1 in the radiation only group compared to the no treatment group. At day 7, all chromosome aberrations were increased except Robertsonian translocations in the radiation only group compared to the no treatment group. These increases in aberrations were significant at the $p<0.05$ level for total, fragments, and gaps but not dicentrics or Robertsonian translocations. The same comparison on day 30 showed that total, fragments, gaps, and Robertsonian translocations were increased in the radiation only

group. These increases were significant for all aberrations categories (Table 2). Fragments followed by gaps were the most common aberration seen in the radiation and RAD>Res groups on days 1, 7, and 30 (Table 1).

TABLE 1
Mean Chromosome Aberrations per Bone Marrow Metaphase Cell for Each Mouse Group at the Designated Time

	Total	Fragments	Gaps	Dicentrics	Robertsonian
No treatment					
Day 1*	0.20±0.55	0.06±0.28	0.07±0.38	0.01±0.11	0.06±0.25
Day 7	0.20±0.55	0.06±0.28	0.07±0.38	0.01±0.11	0.06±0.25
Day 30*	0.14±0.45	0.02±0.15	0.04±0.27	0.01±0.09	0.07±0.25
Resveratrol					
Day 1*	0.12±0.39	0.02±0.18	0.04±0.26	0.02±0.13	0.03±0.18
Day 7	0.12±0.39	0.02±0.17	0.06±0.27	0.00±0.06	0.04±0.19
Day 30*	0.07±0.27	0.01±0.13	0.02±0.15	0.00±0.06	0.03±0.18
Radiation					
Day 1*	2.90±3.04	2.12±2.65	0.33±0.70	0.28±0.71	0.17±0.50
Day 7	0.48±0.76	0.26±0.58	0.14±0.41	0.02±0.15	0.06±0.25
Day 30*	0.51±0.87	0.22±0.61	0.13±0.55	0.01±0.11	0.14±0.39
RAD>Res					
Day 1					
2 hrs	1.00±1.38	0.72±1.29	0.19±0.49	0.02±0.14	0.08±0.29
Day 7					
2 hrs	0.34±0.63	0.08±0.33	0.10±0.33	0.04±0.21	0.11±0.33
2 days+	0.30±0.62	0.09±0.34	0.14±0.43	0.01±0.09	0.06±0.23
Day 30					
2 hrs	0.21±0.44	0.05±0.24	0.07±0.27	0.04±0.19	0.05±0.21
2 hrs+	0.23±0.47	0.05±0.21	0.04±0.21	0.05±0.21	0.09±0.29
2 days+	0.37±0.76	0.14±0.48	0.12±0.39	0.02±0.13	0.10±0.32

Notes: Values are means ± SD for each group of mice based on 250 cells counted. Total chromosome aberrations include fragments, gaps, dicentrics, and Robertsonian translocations. Resveratrol was given as a single dose 2 hours post-irradiation (RAD>Res 2 hrs) or initiated 2 hours (RAD>Res 2 hrs+) or 2 days (RAD>Res 2 days+) after irradiation and continued to the time of bone marrow harvest. * indicates control group values previously reported by the authors (6).

A series of Wilcoxon rank-sum tests were performed to determine which pairs of treatment groups on the same day and which pairs of days for the same treatment group had significantly different mean numbers of total aberrations, fragments, gaps, dicentrics, and Robertsonian translocations at the $p<0.05$ level of significance. When comparing total chromosome aberration frequencies found in the single gavage dose group (RAD>Res 2 hrs) and both continued groups (RAD>Res 2 hrs+ and RAD>Res 2 days+) with the respective radiation groups at days 1, 7, and 30 several significant differences were found (Table 3 and Figure 2).

TABLE 2
Statistical Comparison Between Mouse Groups and Chromosome Aberrations at Each Time

		Wilcoxon rank-sum tests ($p<0.05$)
Event	**Day**	Radiation vs. No Treatment
Total	1	√*
	7	√
	30	√*
Fragments	1	√*
	7	√
	30	√*
Gaps	1	√*
	7	√
	30	√*
Dicentrics	1	√*
	7	ns
	30	ns*
Robertsonian translocations	1	√*
	7	ns
	30	√*

Notes. The √ indicates a significant difference ($p<0.05$) was found and ns indicates that the comparison was not significant. *control group values previously reported (6).

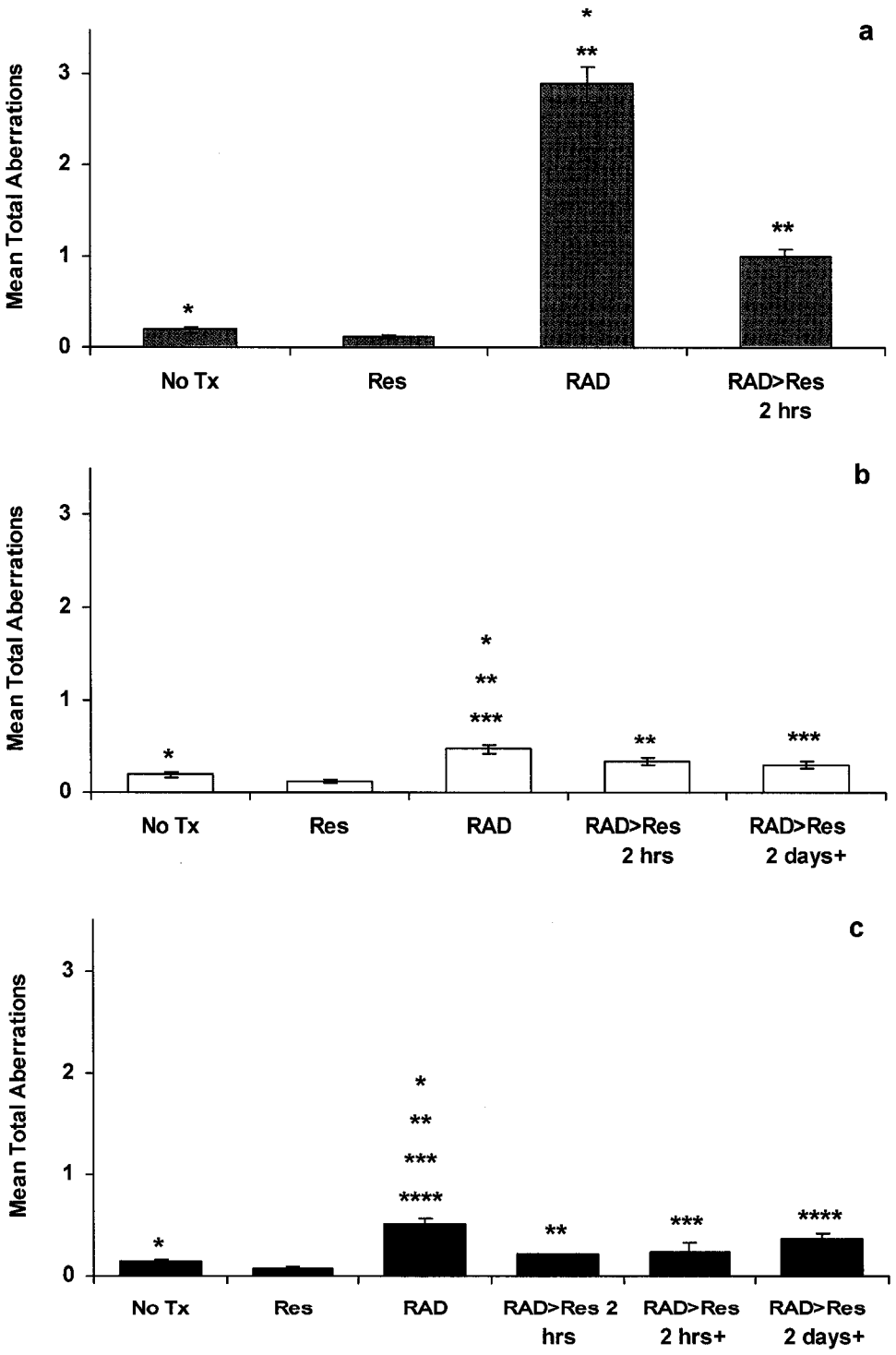

Figure 2a: Mean ± SE total chromosome aberrations per bone marrow metaphase cell for each mouse group are shown for day 1 following 3 Gy whole body γ-irradiation. Statistical comparisons were all $p<0.05$: * – compares the 1 day no treatment (No Tx) group with the 1 day radiation (RAD) group and ** – compares the 1 day radiation

(RAD) group to the 1 day radiation and resveratrol 2 hours post-irradiation (RAD>Res 2 hrs) group for mean total aberrations.

Figure 2b: The mean ± SE total chromosome aberrations per bone marrow metaphase cell for each mouse group at 7 days post-irradiation are shown. Statistical comparisons were all $p<0.05$: * – compares the 7 day no treatment (No Tx) group with the 7 day radiation (RAD) group; ** – compares 7 day radiation (RAD) group to the 7 day radiation followed by resveratrol 2 hours post-irradiation (RAD>Res 2 hrs) group; and *** – compares the 7 day radiation (RAD) group to the 7 day radiation followed by resveratrol 2 days post-irradiation and continued (RAD>Res 2 days+) group.

Figure 2c: Mean ± SE total chromosome aberrations per bone marrow metaphase cell for each mouse group at 30 days post-irradiation are shown. Statistical comparisons were all $p<0.05$: * – compares the 30 day no treatment (No Tx) group with the 30 day radiation (RAD) group; **– compares the 30 day radiation (RAD) group to the 30 day radiation followed by resveratrol 2 hours post-irradiation (RAD>Res 2 hrs) group; *** – compares the 30 radiation (RAD) group to the 30 day radiation followed by resveratrol 2 hours post-irradiation and continued (RAD>Res 2 hrs+) group; and **** – compares the 30 day radiation (RAD) group to the 30 day radiation followed by resveratrol 2 days post-irradiation and continued (RAD>Res 2 days+) group.

Comparing resveratrol administered as a single gavage dose 2 hours after whole body irradiation (RAD>Res 2 hrs) to the radiation only group showed a statistically significant ($p<0.05$) reduction in the mean total chromosome aberrations, fragments, gaps, dicentrics, and Robertsonian translocations at days 1; total chromosome aberrations, fragments, and dicentrics at day 7; and total chromosome aberrations, fragments, and Robertsonian translocations at day 30 (Table 3). In addition, a statistically significant ($p<0.05$) reduction in mean total chromosome aberrations, fragments, and dicentrics were seen at 30 days in mice receiving a gavage dose of resveratrol at 2 hours post-irradiation and continued mixed in the drinking water (RAD>Res 2 hrs+) when compared to the radiation only group at the 30 day end point (Table 3). In the mouse groups receiving resveratrol in the drinking water starting 2 days after whole body irradiation (RAD>Res 2 days+), there was a significant ($p<0.05$) reduction in total chromosome aberration

frequency at the 7 and 30 day time point compared to the respective radiation only group (Table 3).

TABLE 3
Statistical Comparison between Radiation>Resveratrol Treatments and Radiation at Each Time Point

		Wilcoxon rank-sum tests (p<0.05)		
		RAD>Res vs. Radiation		
Event	**Resveratrol treatment**	Day 1	Day 7	Day 30
Total	2 hrs	√	√	√
	2 hrs+			√
	2 days+		√	√
Fragments	2 hrs	√	√	√
	2 hrs+			√
	2 days+		ns	ns
Gaps	2 hrs	√	ns	ns
	2 hrs+			ns
	2 days+		ns	ns
Dicentrics	2 hrs	√	√	ns
	2 hrs+			√
	2 days+		√	ns
Robertsonian translocations	2 hrs	√	ns	√
	2 hrs+			ns
	2 days+		ns	ns

Notes. The √ indicates a significant difference (p<0.05) was found, ns indicates that a significant difference was not present, and the grayed area indicates there were no treatment groups for that end point.

A significant difference in total chromosome aberration, fragments, gaps, dicentrics, or Robertsonian translocation frequency reduction was not (p>0.05) observed at 30 days when comparing the single gavage dose (RAD>Res 2 hrs) with the continued administration of resveratrol in the drinking water (RAD>Res 2 hrs+). In addition, when comparing the groups receiving the initial resveratrol dose at 2 hours post-irradiation (RAD>Res 2 hrs or RAD>Res 2 hrs+) to the group starting resveratrol 2 days post-

irradiation (RAD>Res 2 days+), there was not a significant ($p>0.05$) difference in total chromosome aberrations at days 7 or 30 respectively.

When the mean total chromosome aberration frequencies were compared within a treatment group between time points, it was found that there was a significant ($p<0.05$) difference at days 7 and 30 compared to day 1 following a single dose of resveratrol (RAD>Res 2 hrs); but, not when days 7 and 30 were compared ($p>0.05$). For the groups receiving resveratrol at 2 days post-irradiation (RAD>Res 2 days+), a significant difference ($p>0.05$) was not found when comparing days 7 and 30.

Interestingly, when comparing the treatment groups receiving resveratrol following radiation with the previously published (6) groups receiving resveratrol (100 mg/kg) before radiation (3 Gy), it was found that there was a significant ($p<0.05$) difference between the single dose group (RAD>Res 2 hrs) at 1 day but not at 30 days. At day 1, mice receiving resveratrol before radiation had a higher frequency of chromosome aberrations than the mice receiving resveratrol after radiation (1.40 ± 1.92 compared to 1.00 ± 1.38). For the 2 days post-irradiation start group (RAD>Res 2 days+), there was a significant ($p<0.05$) difference at 30 days compared to the resveratrol initiated before radiation (0.23 ± 0.47 compared to 0.18 ± 0.47 respectively). The comparison was borderline ($p\sim0.05$) at 30 days when comparing the continued dosing starting at 2 hours (RAD>Res 2 hrs+) with mice receiving resveratrol before irradiation.

The No Tx and RES groups (Figure 3 and Table 4) had the fewest mean micronuclei per binucleate cell (0.009 ± 0.96 and 0.017 ± 0.13 respective). As expected, the RAD group (0.057 ± 0.23) had an increased mean number of micronuclei compared to the No Tx and RES groups. RAD>Res had 0.065 ± 0.25 mean micronuclei and

Res+RAD had the highest mean number of micronuclei (0.093 ± 0.29). When comparing the RAD group to the Res+RAD group the p-value was p=0.0005 and to the RAD>Res group the p-value was 0.5746. The comparison of Res+RAD and RAD>Res was p=0.0338.

Discussion

Our results indicate that resveratrol (100 mg/kg) administered 1) as a single dose 2 hours (RAD>Res 2 hrs) after 3 Gy whole body γ-radiation with bone marrow harvested on days 1, 7, and 30, 2) as a bolus 2 hours after 3 Gy whole body irradiation and continued daily mixed in the drinking water (RAD>Res 2 hrs+) until bone marrow

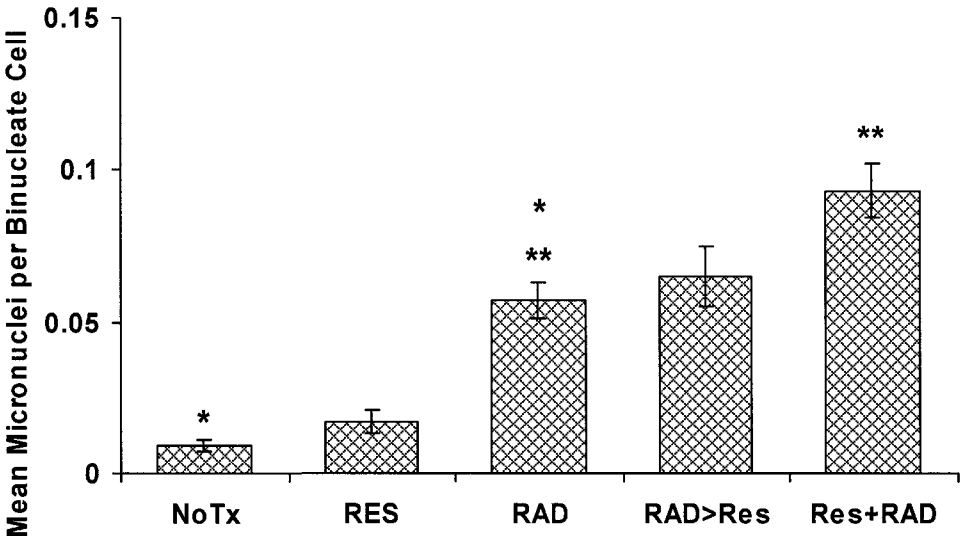

Figure 3: Mean micronuclei ±SE per binucleate bone marrow cell 24 hours after whole body γ-radiation and 18 hours of incubation with cytochalasin B for each treatment group. There were 1500 binucleate cells counted in the no treatment (No Tx) and radiation (RAD) groups, 1000 in the resveratrol (RES), and resveratrol 2 hours after radiation (RAD>Res) groups, and 600 in the resveratrol initiated 2 days before radiation (Res+RAD) group. Statistically significant comparisons were * – compares No Tx group with the RAD group; and ** – compares the RAD group with the Res+RAD group.

harvest at 30 days, and 3) mixed in the drinking water starting 2 days (RAD>Res 2 days+) after 3 Gy whole body γ-irradiation with bone marrow harvest at 7 and 30 days all reduce the mean total chromosome aberration frequency observed in bone marrow metaphases at each of the respective time points when compared to the respective radiation only groups. When resveratrol was given as a single dose (RAD>Res 2 hrs),

TABLE 4
Mean Micronuclei in Each Treatment Group

	NoTx	RES	RAD	RAD>Res	Res+RAD
Mean±SD	0.009±0.96	0.017±0.13	0.057±0.23	0.065±0.25	0.093±0.29

Notes. For each group 500 binucleate cells were scored per mouse except for the Res+RAD group where 200 binucleate cells were scored per mouse. The mean ± SD is shown here.

the mean total chromosome aberration frequency was reduced 65.5% at 1 day, 29.2% at 7 days, and 58.8% at 30 days when compared to the respective radiation only groups. A 54.9% reduction was seen at 30 days in the group receiving resveratrol starting 2 hours post-irradiation and continuing (RAD>Res 2 hrs+); there was a 37.5% reduction at 7 days and a 27.5% reduction at 30 days when resveratrol was started 2 days after radiation and continued (RAD>Res 2 days+) (Figure 2). This compares to the decrease of 51.7% at 1 day and 64.7% at 30 days previously reported for mice receiving resveratrol before radiation compared to the radiation only group (6).

These results show that resveratrol given as a single dose (RAD>Res 2 hrs) was as effective at reducing chromosome aberrations as resveratrol started by gavage 2 hours after radiation and continued (RAD>Res 2 hrs+) to the 30 day end point. Further, a single dose of resveratrol (RAD>Res 2 hrs) was as effective as resveratrol initiated 2 days after radiation and continued (RAD>Res 2 days+) to the 7 and 30 day end points.

Resveratrol started 2 days after radiation and continued (RAD>Res 2 days+) was as effective as resveratrol initiated 2 hours after radiation and continued (RAD>Res 2 hrs+) at the 30 day end point. Interestingly, resveratrol initiated before irradiation (1.40 $\pm$1.92) (6) was less effective ($p<0.05$) at reducing chromosome aberration frequency than a single dose of resveratrol initiated 2 hours after radiation (RAD>Res 2 hrs) at 1 day; however, at 30 days there was no statistical difference.

The specific mechanism responsible for the ability of resveratrol to reduce the frequency of chromosome aberrations was not evaluated in this study. However, it is possible to rule out an antioxidant effect as the predominant protective mechanism based on the results reported here. First, oxidative damage occurs at the time of irradiation and those damaging oxidative species are short lived. Second, it takes time for resveratrol to be absorbed, undergo tissue distribution, and to induce other antioxidants *in-vivo*. For example, maximal induction of catalase mRNA occurred at 24 hours and, over a 72 hour period, cellular catalase and glutathione S-transferase increased 2-fold and 2.5-3-fold respectively in rat aortic smooth muscle cells treated with 100 μM resveratrol (12). Third, the reduction in mean total chromosome aberrations when resveratrol was initiated prior to irradiation was less than those obtained when resveratrol was initiated post-irradiation (6). Therefore, it is reasonable to speculate that other cellular mechanisms besides the antioxidant effect are responsible for the reduction in chromosome aberrations.

Other important points to address are that resveratrol has been shown to sensitize cancer cells to ionizing radiation *in-vitro* (28-30) and that cancer cells and normal cells are affected by different resveratrol concentrations (31-33). For example, resveratrol

exposure inhibited proliferation of 4 leukemia cells lines (mouse 32Dp210 and L1210; and human U937 and HL-60) with an IC_{50} for each cell line of 18, 20, 26, and 34 μM respectively. The IC_{50} for normal bone marrow progenitor cells was 59 μM. Long-term resveratrol (80 μM) exposure reversibly inhibited clonal growth of normal hematopoietic progenitor cells but at a higher IC_{50} compared to the leukemia cell lines (33). Another study showed the IC_{50} for resveratrol-induced apoptosis in the human leukemia cell lines THP1, HL60, U937, and WSU-CLL (5.07, 15.72, 16.09, and 42.76 μM) was also lower than the IC_{50} for normal human bone marrow cells (60 μM) (33). Since resveratrol has similar effects on normal and abnormal cells but at markedly different concentrations, it is possible that the 100 mg/kg dose achieves bone marrow resveratrol concentrations that are at or above the IC_{50} for normal bone marrow cells.

Resveratrol has been shown in leukemia cells to induce cell cycle arrest in S phase and/or at the G2/M transition at concentrations of 15-20 μM, 30 μM, and 100 μM (31,34,35) and normal human lymphoblastoid cells in late G1 or early S phase at 100 μM concentration (36). Depending on how significantly the cell cycle is arrested in the damaged cells, a larger proportion of damaged cells could be present at the day 1 end point in mice receiving resveratrol before radiation compared to those mice with resveratrol initiated post-irradiation. Induction of apoptosis by resveratrol in seriously damaged cells at concentrations of 10-200 μM (14,17,37) would facilitate a reduction in the observed frequency of chromosome aberrations in all groups receiving resveratrol but occurs in higher levels at 48-72 hours.

Another consideration is that resveratrol, as summarized by Gatz and Wiesmuller (38), has a variety of nuclear activities that play a role in DNA repair including telomere

maintenance, DNA relaxation, DNA synthesis, base excision repair, double strand break repair, and chromatin remodeling. Therefore, studies exploring the significance of cell cycle arrest, apoptosis, and DNA repair in these mice are warranted to further define the contribution of each to the reduction of bone marrow cell chromosome aberrations.

The *in-vivo* effects of resveratrol are dependent on the tissue levels achieved with the selected resveratrol dose. The dose used in this study (100 mg/kg) was expected to produce tissue concentrations consistent with concentrations that have been shown to be active on molecular targets *in-vitro*. The pharmacokinetics of resveratrol in mice has been studied and some tissue concentrations reported. Resveratrol doses in reported studies have ranged from 0.6 mg/kg to 1500 mg/kg with either oral or IP administration (39-42). One study using an oral dose of 50 mg/kg achieved a liver and kidney concentration of 25-30 μM with resveratrol conjugates still present at the final 6 hour time point (43). With a single oral dose of 4000 mg/kg, resveratrol sulfate and glucuronide conjugates were still detectable at 24 hours. An increase in these conjugates was found between the 4 and 8 hour time points and was attributed to enterohepatic recirculation (44). These studies indicate that resveratrol conjugates are present for long periods following oral or IP administration. With this in mind, it is possible for resveratrol to have cellular effects over a prolonged time period *in-vivo* after the initial administration.

Toxicity studies using extremely large resveratrol doses (2000-4000 mg/kg mice and 3000 mg/kg rats) have demonstrated renal toxicity (44,45), while lower daily doses of 20 mg/kg dose in rats did not (46). However, in studies investigating the potential for genotoxicity, micronuclei were detected in mouse lymphoma cells (L5178Y) and Chinese

hamster V79 cells at 1-60 μM resveratrol concentrations (47); micronuclei, sister chromatid exchanges, and chromosome aberrations detected in a Chinese hamster lung cell line at 2.5-20 μg/ml (48); and sister chromatid exchanges in the Chinese hamster cell line SPD8/V79 at 0.625-5 μg/ml (49). These results should be interpreted with caution because, as stated above, cancer cells have been shown to have an increased sensitivity to resveratrol compared to normal cells and the Chinese hamster V79 cells have been shown to have a disruption of normal DNA damage response pathways (50). The inability of these cells to properly respond to DNA damage could reduce their survival following treatment with resveratrol in a fashion similar to that observed with other abnormal cells. Resveratrol did reduce the cell survival of Chinese hamster lung cells during the 24 and 48 hour end points (48). In support of the differential effects of resveratrol on normal and abnormal cells, chromosome aberration frequency was not increased in normal bone marrow cells above background levels at a daily dose of 100 mg/kg after 30 days of continuous administration in the study reported here.

In addition, there was not an increase in micronuclei formation in the RES group compared to the No Tx group in the study reported here. However, there was an increase in the RAD, RAD>Res, and Res+RAD groups compared to the No Tx group. The mean number of micronuclei was not significantly different between the RAD and RAD>Res groups but was when comparing RAD to Res+RAD and RAD>Res to Res+RAD. This observation implies that resveratrol at 100 mg/kg initiated before radiation sensitizes the cells to radiation damage. Yet, our previously reported results indicate that there is a reduction in observed radiation-induced chromosome aberrations in the metaphase bone marrow cells when resveratrol is initiated prior to irradiation (6).

Bone marrow is a complex grouping of different cell type populations including leukocytes, erythrocytes, megakaryocytes, and stromal cells at varying stages of maturation. Exact kinetics of each of these bone marrow cell populations in the mouse is unclear; however, studies have been done evaluating bone marrow cells and bone marrow stromal cells following irradiation (51-54). Irradiated bone marrow cells have been shown to have changes in the number of bone marrow cells including stem cells after 1.5 or 2.6 Gy irradiation (55); gene expression resulting in reduced entry of cells into S-phase and in G2/M arrest following 6.5 Gy irradiation (41); alterations in the bone marrow stromal cell compartment detectable *in-vitro* 6 months after 2 Gy irradiation (54); and reduced bone marrow stromal cell capacity for self-renewal and altered blocking of apoptosis in attached hematopoietic stem cells following irradiation (53).

The effect of radiation on bone marrow cellularity is of particular interest for the study reported here. Following a 2.6 Gy irradiation, the bone marrow cell count in the femur decreased from 16.5×10^6 to 3×10^6 at 48 hours. By day 6 normal levels of bone marrow cells were found and the marrow became hypercellular at 7-12 days (55). Assuming that the most damaged cells are lost initially, this study shows that the most significant loss of bone marrow cells occurs in the initial 48 hours following 2.6 Gy irradiation. In accordance with this, the study reported here found that bone marrow cells from the day 1 radiation only group had the highest frequency of chromosome aberrations and aberration frequencies were lower at days 7 and 30 (Table 1). Cells that have the potential for repair or altered ability to undergo apoptosis may persist and the relatively slower turnover of more mature bone marrow cells may contribute to the

continued presence of cells with chromosome aberrations 30 days after 3 Gy whole body irradiation.

The results reported here show that resveratrol has the ability to reduce chromosome aberration frequencies in bone marrow cells when it is given after radiation exposure. At the resveratrol dose used in this study, it appears that a single gavage dose of resveratrol 2 hours (RAD>Res 2 hrs) after radiation is statistically ($p<0.05$) superior to the same dose of resveratrol administered before radiation at 1 day but there was no difference at 30 days. Since resveratrol has been shown in other studies to have a radiosensitizing effect *in-vitro* at differing exposure times and IC_{50} for each cancer cell line (28-30), it is possible that the 100 mg/kg dose used in the previously reported study (6) is reaching high enough bone marrow concentrations or achieving long enough exposure time to increase the radiation sensitivity of normal bone marrow cells. This would be consistent with the increase in micronuclei observed in the present study in the Res+RAD group compared to the RAD>Res group. However, it is important to recognize that the resveratrol initiated before radiation can induce cell cycle arrest in the G1/early S phase in normal human lymphoblastoid cells (AHH-1) (36) giving the cells an intermediate sensitivity to radiation-induced damage. The cell cycle arrest is then likely followed by apoptosis that increases over a 72 hour period following resveratrol exposure (29). This cell cycle arrest followed by apoptosis would account for why there is a reduction in chromosome aberrations regardless if the resveratrol is initiated prior to or after irradiation, why the post-irradiation start for resveratrol has a lower frequency of chromosome aberrations, and why the frequency of chromosome aberrations are so similar at the 30 day end points.

With these points in mind, identification of an optimal dose for radioprotection is essential for clarifying this relationship before resveratrol can be used for clinical applications. In regard to the comparable results observed in the 2 hour initiation single dose (RAD>Res 2 hrs) and continued resveratrol groups (RAD>Res 2 hrs+ and RAD>Res 2 days+), it is possible that resveratrol exerts its optimal chromosome aberration reductive effect during a defined post-irradiation time period when apoptosis is the prevalent mechanism for eliminating damaged and unrepairable cells.

Future studies will define the optimal dose of orally administered resveratrol and the point of optimal initiation of resveratrol relative to radiation exposure. In addition, cell cycle arrest and apoptosis studies will further define the time course relationships that underlie the interesting observations of this study and the previously reported results. These studies will facilitate a better understanding of the dose and cell line dependent ability of resveratrol to act as both a radiosensitizer and a radioprotector.

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- Chapter 4 -

Resveratrol and Muscadine Grape Reduce Radiation-induced Loss of the PU.1 Gene in Mouse Bone Marrow Cells

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Abstract

Resveratrol, a polyphenolic stilbene compound with antioxidant, anti-inflammatory, and anticarcinogenic effects, with toxicity only at extreme doses, has been widely studied. In the study reported here, we evaluated the ability of orally administered resveratrol and a muscadine grape extract to reduce PU.1 gene deletion in irradiated mouse bone marrow cells. The PU.1 protein is a transcription factor that plays an important role in bone marrow stem cell renewal and differentiation of the granulocyte cell line. CBA/CaJ mice were divided into six main groups: 1) no treatment, 2) resveratrol (100 mg/kg) only, 3) radiation (3 Gy γ radiation) only, 4) resveratrol (100 mg/kg) started before radiation (3 Gy), 5) radiation (3 Gy) followed by initiation of resveratrol (100 mg/kg), and 6) muscadine grape (5.73 μg/kg total *trans*-resveratrol) followed by irradiation (3 Gy). Resveratrol administration was initiated 2 days before radiation with a third dose given 30 minutes prior to radiation. In the groups receiving radiation before resveratrol, the resveratrol was started 2 hours or 2 days post-irradiation. Bone marrow was harvested at 1 and 30 days following a single whole body radiation exposure. A statistically significant ($p<0.0001$) reduction in loss of the PU.1 gene was observed in metaphase bone marrow cells at each time point after irradiation in the resveratrol (pre-irradiation and 2 hours post-irradiation) treatment groups and muscadine grape extract treatment group compared to the radiation only group on day 1 and 30. Our results are in accord with our previously reported studies showing a reduction in radiation-induced chromosome aberrations in mouse metaphase bone marrow cells and further support the use of resveratrol as a radioprotectant with the potential for widespread application.

Introduction

There is a growing interest in the study and use of dietary compounds, like resveratrol (*trans*-3,5,4'-trihydroxystilbene), for disease management including use for radioprotection (1-3). Resveratrol has been shown to have effects on a large number of molecular targets including those that influence cell cycle progression, apoptosis, signal transduction, and inflammation (4,5). Interestingly, there is a long history of use of plants containing resveratrol including the Chinese herbal preparation, Ko-jo-kon with *Polygonum cuspidatum*, and the Ayurvedic medicine, darakachsava with fermented grape, that mirrors current research findings with resveratrol's anti-inflammatory actions, cardioprotective properties, and its anti-aging effects (6-8). Resveratrol has been identified in over 72 different plants including grapes, peanuts, blueberries, bilberries, cranberries, lingonberries, and the weed *Polygonum cuspidatum* (6,9-12).

We have previously shown that resveratrol significantly reduces the frequency of radiation-induced chromosome aberrations observed in metaphase bone marrow cells when administered prior to a single whole-body dose of γ-radiation (3). This property of resveratrol is important because ionizing radiation is known to create DNA damage that can lead to mutagenesis and carcinogenesis depending on the total dose, dose rate of delivery, and animal species (13-15). Radiation exposure can occur from both occupational and therapeutic sources. In addition, increasing survival following radiation therapy for cancer has led to increased risk of therapy-induced secondary cancers including acute myeloid leukemia (t-AML) (16-17). AML is a disease that can have a fulminating onset and can be rapidly fatal without appropriate therapy (18). Furthermore, t-AML carries a worse prognosis compared to de novo AML (19). Therefore,

identification of cost effective, nontoxic methods to reduce the incidence of chromosome aberrations that potentially lead to t-AML is important. While a range of chromosome abnormalities have been identified in humans associated with AML, some authorities report increased incidence of PU.1 (*Sfpi1*) gene mutations in 7% of human AML patients (20) however, other studies have not confirmed this finding (21). Interestingly, even though the PU.1 gene has not always undergone mutation, a decrease in PU.1 expression resulting from inactivity of other transcription factors has been reported associated with human AML (22-24).

The PU.1 protein is an ETS transcription factor that is restricted to blood cells. PU.1 is considered a transcriptional master regulator of myeloid and lymphoid cells which is evidenced by the presence of a PU.1-binding motif in the regulator sequences of almost all myeloid-specific and many lymphoid-specific genes. In addition, PU.1 is detectable in hematopoeitic stem cells (HSC), common myeloid progenitor cells (CMP), common lymphoid progenitor cells (CLP), and in mature myeloid cells. PU.1 is important for the HSC self repopulation and differentiation into CMP and CLP. While other transcription factors play a role in maturation and differentiation of leukocytes in the bone marrow, it is the response to graded levels of the PU.1 protein that is essential for differentiation of the granulocyte/monocyte progenitor cells (GMP) into granulocytes or monocytes (25). Increased PU.1 protein favors monocyte commitment of GMP cells (26).

The CBA mouse is an ideal model for studying radiation-induced AML. This mouse strain has a low incidence of background AML (<1%) and develops AML following irradiation. A single 3 Gy dose of ionizing radiation provided the maximum

incidence of AML (25%) in CBA mice (27,28). A genetic instability has been identified on chromosome 2 in the D-E region in CBA mice with radiation-induced AML that includes the PU.1 gene (29,30). Interestingly, the development of AML in these mice results from a combination of the deletion of a single PU.1 gene allele and a missense mutation in the remaining PU.1 allele in the DNA-binding domain (30,31). This missense mutation results in an inability of the PU.1 protein to bind DNA properly (30).

In the study reported here, the ability of resveratrol initiated before or after whole-body γ-radiation to reduce the loss of the PU.1 gene in bone marrow cells was determined 1 and 30 days post-irradiation in the CBA mouse. In addition, the impact of a muscadine grape extract on PU.1 gene loss in bone marrow cells was investigated at day 1 post-irradiation. The ability of resveratrol to modulate the radiation-induced loss of the PU.1 gene can provide an important opportunity to gain valuable insights into potential clinical applications for resveratrol as a single agent. More importantly, the ability of a muscadine grape extract containing food quantities of resveratrol to reduce the radiation-induced loss of the PU.1 gene in bone marrow cells would provide evidence that μM concentrations of bioactive substances found in food are capable of exerting beneficial cellular and molecular effects.

Materials and Methods

Animals

Eight to nine week old, male CBA/CaJ mice (24.7±0.7 g) obtained from Jackson Laboratory were used in this study. Mice were allowed to acclimate for approximately one week prior to use. The mice were housed and maintained according to the approved

standards in the Laboratory Animal Resources facility at Colorado State University (CSU). The Animal Care and Use Committee at CSU approved animal handling and experimental protocols.

Treatment groups

A total of 60 9-10 week old, male CBA/CaJ mice were divided into 2 sets (1 day and 30 days) of 6 groups each, 5 mice each, as follows: 1) no treatment (No Tx), 2) resveratrol (RES) only (100 mg/kg), 3) radiation (RAD) only (3 Gy), 4) Res+RAD (100 mg/kg and 3 Gy), 5) RAD>Res (3 Gy and 100 mg/kg), and 6) Mus>RAD (5.73 μg/kg total *trans*-resveratrol in muscadine (Mus) and 3 Gy). One and 30 days following whole body irradiation, bone marrow from each mouse was individually harvested, processed, slides prepared and metaphase chromosome aberrations scored as described below.

Radiation

For radiation exposure, mice were placed as a group of five into a well ventilated plexiglass container. A total dose of 3 Gy γ-radiation, at a dose rate of 1.13 Gy/min, was delivered using a ^{137}Cs irradiator (J.L. Shepherd model 81-14).

Resveratrol Dose and Administration

Trans-resveratrol (Sigma-Aldrich) was dissolved in 100% ethanol to a concentration of 50 mg/ml. Prior to gavage, the resveratrol/ethanol dose was diluted as follows: 0.05 ml of resveratrol (50 mg/ml) in 0.2 ml distilled water for a final concentration of 10 mg/ml. Individual mice in each designated group received a resveratrol dose of 100 mg/kg administered by gavage every 24 hours for 2 days prior to irradiation, and again on the day of irradiation, 30 minutes prior to radiation exposure for the Res+RAD groups while the RAD>Res 2 hrs groups received 1 gavage dose (100

mg/kg) 2 hours after irradiation, and the RAD>Res 2 days+ group, resveratrol was started 2 days after irradiation. For RAD>Res 2 days+ mice in the 30 day group received resveratrol daily with the resveratrol/ethanol mixture added to the drinking water at a rate of 50 mg resveratrol per 100 ml and continuing until the final day of the study period. The resveratrol stock and resveratrol water combination were protected from light by covering the container with aluminum foil. The resveratrol water combination was regularly changed.

Dosing for the muscadine grape extract was based on the total *trans*-resveratrol content determined by previous analysis using tandem mass spectrometry (MS/MS) coupled with high performance liquid chromatography (HPLC). The calculated amount of the muscadine grape extract was mixed with ethanol to volume of 0.05 ml and then mixed with 0.2 ml of distilled water to deliver 5.73 μg/kg of total *trans*-resveratrol. This dose was administered by gavage to each mouse in the Mus+RAD group every 24 hours for 3 days with the third dose given 30 min prior to irradiation.

Muscadine Grape Extract Resveratrol Content

The muscadine grape extract was prepared by adding 3 g of muscadine grape powder (Muscadine Naturals, Inc, Lewisville, NC) to 10 ml of 100% ethanol, covered with aluminum foil, and repeatedly shaken. Approximately 24 hours later, any material in the suspension that could pass through a 22 ga needle was removed and placed at -20°C until used for analysis of total *trans*-resveratrol or administered to the indicated group. The muscadine grape extract was used within 48 hours of removal from the suspension.

Nine muscadine grape extract samples were analyzed using MS/MS (MDS Sciex 3200 Q TRAP®, MDS Analytical Technologies, Concord, Ontario, Canada) coupled with HPLC (Agilent 1200 Series Binary Pump SL, Foster City, CA). Each muscadine grape extract was diluted 1:5 in methanol, an internal standard was added (up to 100 ng/ml), the solution was filtered with a 2 μm filter, and then injected in the liquid chromatography/mass spectrometer. The *trans*-resveratrol and *trans*-piceid (*trans*-resveratrol-3-O-D-glucoside) standards were obtained from Sigma-Aldrich. Following quantitative analysis for both compounds, the mean ± SD were derived for both compounds and the combined total.

Bone Marrow Collection

Colchicine (0.1 ml of a 0.5% solution) was administered by intraperitoneal (IP) injection to each mouse 1 hour prior to bone marrow collection. Bone marrow was flushed from each femur, tibia, and radius with PBS. The PBS bone marrow mixture was centrifuged at 1000 rpm for 5 min at 4°C. The cell concentrate was then placed into an incubation mixture (9 ml 0.75 M KCl, 1 ml EDTA trypsin, 200 μl colcemid), mixed, and incubated for 30 min at 37°C. Following incubation, 5 ml fresh fixative (3:1 methanol:acetic acid) was added, mixed, and centrifuged at 1000 rpm for 5 min. Cells were washed with fresh fixative two more times. The supernatant was decanted, cells resuspended in a small amount of fixative, and cells were dropped onto clean, wet microscope slides and allowed to dry.

PU.1 Gene Fluorescence in Situ Hybridization

Slides containing metaphase bone marrow cells were coded and processed for fluorescence in situ hybridization (FISH). The slides were placed in 70% formamide at

70°C for 1 minute and 45 seconds to denature followed by am ethanol dehydration series of 2 min intervals: 1) 75% ethanol at -20°C, 2) 75% ethanol at room temperature, 3) 85% ethanol at room temperature, and 4) 100% ethanol at room temperature. Once air dried, the slides were warmed to 37°C for 10 minutes before the probe mixture and cover slips were applied. The slides were placed into a hybridization slide chamber (Corning) and held at 37°C for approximately 24 hours. Following this incubation, the cover slips were removed and the slides were washed 2 times in each solution for 4 min at 42°C: 1) 50% formamide (Fisher Scientific) in 2XSCC (saline-sodium citrate buffer) at pH 5.8, 2) 2XSCC pH 7.4, and 3) PN buffer. DAPI DNA counterstain (Vector Laboratories) and a glass cover slip were placed on the slides and the coded slides were blinded for scoring of PU.1 gene loss.

Just prior to use, the PU.1 FISH hybridization mixture for each slide was prepared by mixing 4 μl mouse Cot-1 DNA (Invitrogen, Carlsbad, CA), 18 μl hybridization mixture containing 10% dextran sulfate (Sigma, St. Louis, MO), 7 μl distilled water, and 1 μl PU.1 probe. These ingredients were thoroughly mixed, heated to 84°C for 10 min and then to 37°C for 20 min before application to the slides as described above.

The PU.1 FISH probe was prepared using the BAC clone RP23-263H8 (BACPAC Resources, Children's Hospital, Oakland Research Institute, Oakland, CA) which contains the mouse PU.1 gene. Identity of the BAC clone was confirmed by the PCR amplification of a segment of PU.1 sequence (GCCCCGGATGTGCTTCCCTTATCAAACC and TGCCTCGGCCCTGGGAATGTC) with primers. The BAC DNA was extracted using the QIA filter Plasmid Maxi kit (QIAGen). This BAC DNA (1μg) was labeled with Alexa Fluor® 594-5-dUTP

(Invitrogen) using a nick translation kit (Roache Applied Science) according to the manufacturer's instructions, except with an increased incubation period of 150 min. The labeled probe was precipitated in 2 volumes of ethanol and 1/10 volume of 3M sodium acetate and then resuspended in distilled water. Additional verification of the genomic location of the probe was confirmed by hybridizing the PU.1 probe to metaphase mouse bone marrow cells simultaneously with FITC labeled whole chromosome 2 painting probe (Star-FISH®, Cambio, Cambridge, UK) and determining colocalization at the expected position on chromosome 2.

The normal metaphase bone marrow cell has 4 PU.1 genes; therefore, each of the PU.1 genes will be labeled with the probe giving 4 signals. PU.1 gene loss was determined when identified when 2 probes were seen on only one chromosome in metaphase cells with the full complement of 40 chromosomes (Figure 1). A total of 100 metaphase bone marrow cells from each mouse were scored blind with a total of 500 cells scored for each treatment group.

Statistical Analysis

Statistical analysis consisted of the evaluation of the mean PU.1 gene loss per time point for each treatment group. The data are presented as the mean $\pm$ standard error of mean percent of PU.1 gene loss per bone marrow metaphase cell. Chi-square test was used to evaluate the association between mouse treatments and PU.1 loss.

Results

Exposure to ionizing radiation increased the incidence of PU.1 gene loss in the CBA mice bone marrow cells evaluated in this study when comparing the RAD only

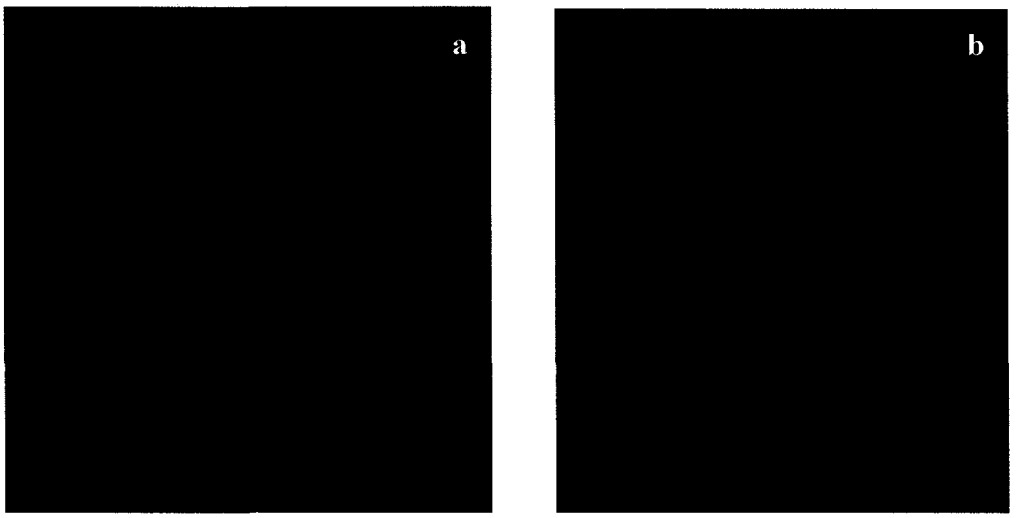

Figure 1. Metaphase bone marrow cells from mice in the no treatment (No Tx) group (a) and the radiation (RAD) only group (b) at day 1 post-irradiation showing hybridization with the PU.1 gene probe. The cell from the No Tx group has the appropriate number of copies (4) of the PU.1 gene while the cell from the RAD group has only 2 copies indicating that the PU.1 gene has been lost from 1 copy of chromosome 2.

group (11.00% and 5.60% PU.1 gene loss at days 1 and 30 post-irradiation respectively) to the No Tx group (0.80% and 1.20%). Resveratrol alone (RES group – 0.80% and 1.20%) did not increase PU.1 gene loss compared to the No Tx group at days 1 and 30 (Table 1 and Figures 2 and 3). When resveratrol was initiated 2 days before radiation in the Res+RAD group the mean PU.1 gene loss was 1.40% on day 1 and 0.40% on day 30. In the RAD>Res 2 hrs group that received a single dose of resveratrol 2 hours after

TABLE 1

Mean Percent PU.1 Gene Loss in Each Treatment Group at the Designated Time Point

	No Tx	RES	RAD	Res+RAD	RAD>Res 2 hrs	Mus+RAD	RAD>Res 2 day+
Day 1	0.80±0.40	0.80±0.40	11.00±1.40	1.40±0.53	1.40±0.50	0.80±0.50	nd
Day 30	1.20±0.49	1.20±0.49	5.60±1.03	0.40±0.28	0.80±-.0.40	nd	1.13±0.05

Note: Values are mean percent ± SE PU.1 gene loss observed in 500 metaphase bone marrow cells in each group at each time point. nd indicates no data available.

radiation, the mean PU.1 gene loss was 1.40% on day 1 and 0.80% on day 30. This compares to the RAD>Res 2 days+ group where resveratrol was initiated 2 days after irradiation and continued to 30 days that had a mean PU.1 gene loss of 1.13%. The mean

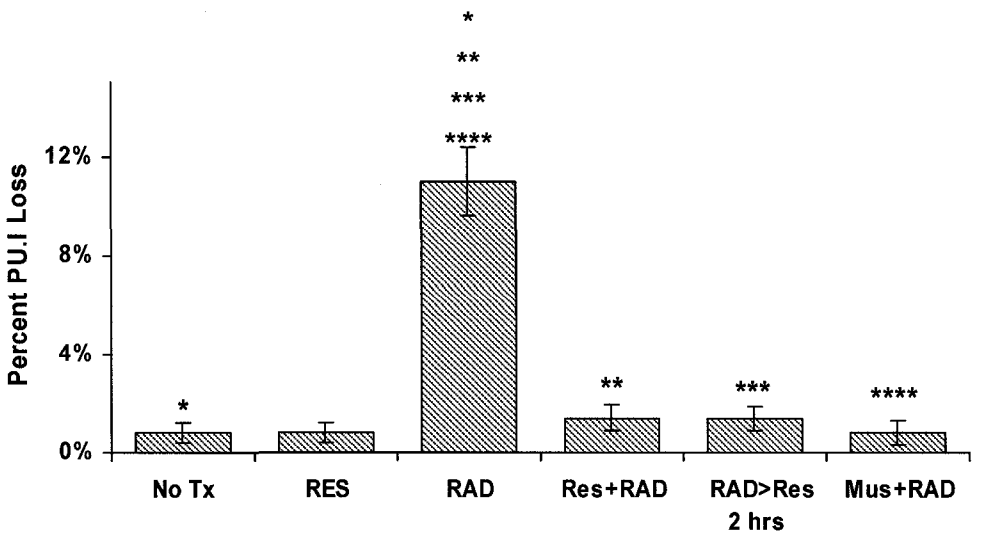

Figure 2. The mean percent loss of 1 copy of the PU.1 gene ± SE in metaphase bone marrow cells as determined on day 1 after whole-body γ-radiation. p-value for significant comparisons $p<0.0001$ * - comparison between the no treatment (No Tx) and the radiation (RAD) only groups, ** - comparison between the radiation (RAD) only and resveratrol given prior to radiation (Res+RAD) groups, *** - comparison between radiation (RAD) and radiation followed by resveratrol 2 hours after irradiation (RAD>Res 2 hrs) groups, and **** - comparison between radiation (RAD) and the muscadine extract administered before radiation (Mus+RAD) groups.

PU.1 gene loss in the mice receiving the muscadine extract (Mus+RAD) starting 2 days before irradiation was 0.80% on day 1. The increase in PU.1 loss in the RAD group compared to the No Tx group was significant at $p<0.0001$ at day 1 and $p=0.0001$ at 30 day. Resveratrol significantly reduced the loss of the PU.1 gene when comparing the pre-irradiation Res+RAD group with the RAD group ($p<0.0001$) at 1 and 30 days; the post-irradiation RAD>Res 2 hrs group with RAD ($p<0.0001$) at 1 and 30 days; the post-irradiation RAD>Res 2 days+ and continued group with the RAD group ($p=0.0029$) at 30 days; and the pre-irradiation Mus+RAD group with the RAD group ($p<0.0001$) at 1 day.

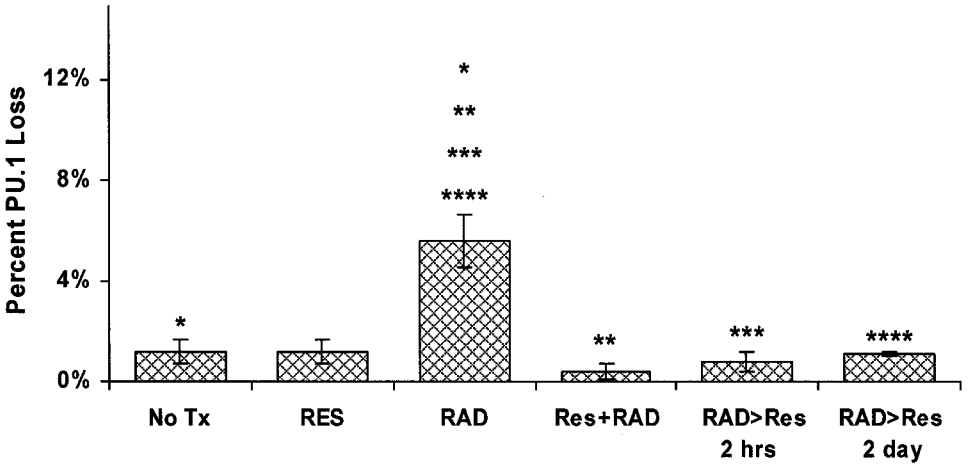

Figure 3. For day 30 following whole-body irradiation, the mean percent loss of 1 copy of the PU.1 gene ± SE in metaphase bone marrow cells. * - $p<0.0001$ when comparing the no treatment (No Tx) and the radiation (RAD) only groups, ** - $p<0.0001$ when comparing radiation (RAD) only and resveratrol started before radiation (Res+RAD) groups, *** - $p<0.0001$ comparing between radiation (RAD) and radiation followed by a single resveratrol dose 2 hours after irradiation (RAD>Res 2 hrs) groups, and **** - $p=0.0029$ comparing between radiation (RAD) only and radiation followed by resveratrol 2 (RAD>Res 2 days+) days after irradiation and continued to 30 days.

The volume of muscadine grape extract administered was based on the total *trans*-resveratrol content. This represents the combined total of *trans*-resveratrol and *trans*-piceid (resveratrol glycoside). The mean ± standard deviation of the total *trans*-resveratrol content was found to be 1790.46 ± 262.46 ng/ml (Table 2).

TABLE 2

Mean Concentration of Resveratrol and Piceid Present in the 3g Muscadine Grape Extract

Resveratrol	Piceid	Total
1277.48 ± 245.14	512.98 ± 99.51	1790.46 ± 262.46

Note: Values represent the mean ± SD in ng/ml for analysis for *trans*-resveratrol and *trans*-piceid content in the muscadine grape extract using 3g muscadine grape mixed with 10 ml of 100% ethanol.

Discussion

Resveratrol at 100 mg/kg administered starting before (Res+RAD) or after (RAD>Res 2 hrs or RAD>Res 2 days+) irradiation significantly reduced radiation-induced PU.1 gene loss in the CBA mouse as seen in metaphase bone marrow cells collected on day 1 and 30 post-irradiation. These results are important because they demonstrate that small molecules like resveratrol can effectively modulate the frequency of radiation-induced chromosome aberrations, even those associated with fragile genomic sites like the one containing the PU.1 gene. Our previous report showed that resveratrol significantly reduces radiation-induced chromosome aberration frequencies in metaphase bone marrow cells from CBA mice at 1 and 30 days post-irradiation (3). When these findings are taken together with the present study, the results imply that resveratrol has the potential to decrease the frequency of abnormalities resulting from radiation in general as well as specifically important genomic sites like the PU.1 gene.

This reduction in radiation-induced loss of the PU.1 gene at day 1 and 30 is likely the result of a combination of resveratrol effects including cell cycle arrest, reduction in survival proteins, and apoptosis induction (4). The direct antioxidant effect and indirect antioxidant effect of resveratrol through induction of superoxide dismutase, catalase, and glutathione (32,33) does not appear to be significant for reducing damage resulting from radiation generated reactive oxygen species because the mean percent PU.1 gene loss was the same whether the resveratrol was started before (Res+RAD) or after (RAD>Res 2 hrs) irradiation at the 1 and 30 day end points. At the 1 day time point post-irradiation in the Res+RAD, cell cycle arrest combined with reductions in survival proteins like survivin and induction of apoptosis likely play a central role in reducing the loss of the

PU.1 gene. A partial early S phase arrest has been shown to occur in IM9 (human myeloma cell line) cells with an accumulation of cells in S phase (72.9% vs 45.4% in control) via ATM check point signaling followed by a reduction of cells in the G_1 and G_2/M phases 24 hours after contact with 30 μM resveratrol (34). In conjunction with this cell cycle arrest a decrease in survivin mRNA has been shown in ST1 (primary adult T cell leukemia) cells in a dose dependent fashion following 10 hours of 20-80 μM resveratrol exposure (35). Survivin is an inhibitor of apoptosis and its decline would contribute to induction of apoptosis. Apoptosis was found in IM9 cells at the 24 and 48 hour time point examined after 10μM resveratrol exposure (34). Consistent with this observation, apoptosis was shown to continue to increase in HCT 116 (human colon adenocarcinoma cell line) cells from 12 hours to the 72 hour end point of the study when the cells were exposed to 25-100 μM resveratrol (36). With the results of these studies in mind, it can be seen that cells exposed to resveratrol and radiation go through a roughly sequential pattern of cell cycle arrest in S phase in which the cell has a prolonged opportunity to repair the radiation-induced chromosome damage. Damaged cells that are unable to be repaired are then induced to undergo apoptosis. Therefore, on day 1 post-irradiation, bone marrow cells from the Res+RAD group had been exposed to resveratrol for approximately 96 hours. Since resveratrol is known to exert effects on normal cells at a higher dose than immortalized or abnormal cells, it can be speculated that these bone marrow cells would be in a heightened state of readiness for DNA repair or apoptosis through priming by resveratrol. Therefore, when these primed cells are exposed to radiation, a more rapid apoptotic response could be anticipated; however, the maximal effects likely would not be seen by the 24 hour post-irradiation end point. It should be

kept in mind that radiation damage also induces apoptosis. Cell cycle arrest is expected to be most prominent process at day 1 post-irradiation in the RAD>Res 2 hrs group with apoptosis contributing to the long term decrease in PU.1 gene loss as seen at day 30 in the RAD>Res 2 hrs and RAD>Res 2 days+ groups.

While resveratrol at 100 mg/kg reduced the radiation-induced loss of the PU.1 gene at both the day 1 and day 30 time points, it is important to recognize that this dose of resveratrol, while not toxic, is too high to be reasonably achieved through the foods like grapes, peanuts, or wine. Therefore, resveratrol as a single agent was compared to resveratrol as found in a muscadine grape extract. Muscadine grape extract was administered based on the total *trans*-resveratrol content and initiated 48 hours before radiation exposure providing a direct comparison with the Res+RAD group. The amount of muscadine grape extract used in this study provides a dose of resveratrol (5.73 μg/kg of total *trans*-resveratrol) that is achievable with a food source.

When this dose of muscadine grape extract was administered before radiation exposure (Mus+RAD) significantly reduced the radiation-induced loss of the PU.1 gene at 1 day after irradiation. PU.1 gene loss was reduced to background levels on day 1 indicating that either resveratrol as a single agent could be used at much lower dose or that resveratrol in combination with the other constituents of the grape matrix provides a synergistic effect that makes the muscadine grape extract more effective than the resveratrol alone. We have determined that resveratrol as a single agent at 5.75 μg/kg reduces radiation-induced mean total chromosome aberration frequencies in metaphase mouse bone marrow cells to the same extent as resveratrol at 100 mg/kg (1.47 ± 1.84 and 1.40 ± 1.92 respectively) (see Chapter 5). With this in mind, it is expected that a dose of

5.75μg/kg of resveratrol would result in the same level of PU.1 gene loss as the 100 mg/kg dose. When comparing Mus+RAD to Res+RAD at 1 day, it can be seen that the muscadine grape extract group had a lower mean percent of PU.1 gene loss (0.80 ± 0.50 and 1.40 ± 0.53 respectively). However, this difference was not statistically significant.

It has been reported that grapes contain over 1600 constituents including resveratrol, piceatannol (*trans*-3,4,3',5'-tetrahydroxystilbene), and pterostilbene (3,5-dimethoxy-4'-hydroxystilbene) (37). Piceatannol and pterostilbene are mentioned here because their basic structure is the same as resveratrol and because they have been shown to have similar molecular effects as resveratrol (4). Interestingly, resveratrol is metabolized to picetannol by the cytochrome P450 enzyme CYP1B1 and CYP 1A2 (38,39). Further, piceatannol, is considered to be more effective than resveratrol in inducing beneficial cellular effects (4).

The results reported here for the Res+RAD group showing a reduction in radiation-induced PU.1 gene loss are consistent with our previous results investigating the effect of resveratrol given before radiation on the frequency of chromosome aberrations in mouse bone marrow cells using a 100 mg/kg dose of resveratrol (3). Additionally, these PU.1 gene loss results are consistent with the chromosome aberration frequency studies examining resveratrol initiated after irradiation (RAD>Res 2 hrs and RAD>Res 2 days+) (see Chapter 3). The effect of administering muscadine grape extract (Mus+RAD) before irradiation on the frequency of chromosome aberrations is consistent with the reduction in PU.1 gene loss seen in the study reported here (see Chapter 5).

Clearly, resveratrol as a single agent or in concentrations found in food, can significantly reduce the radiation-induced loss of the PU.1 gene. This effect is observed

in metaphase bone marrow cells whether the resveratrol is initiated before (Res+RAD and Mus+RAD) or after irradiation (RAD>Res 2 hrs and RAD>Res 2 days+) at 1 and 30 days post-irradiation in the respective groups reported here. These results are significant for a number of reasons. First, since resveratrol has benefits regardless of when it is initiated around the time of radiation exposure, flexibility exists regarding when resveratrol is started relative to the radiation exposure. This provides opportunities to use resveratrol prior to a known radiation exposure or following an unexpected exposure. Second, because the concentration of resveratrol found in the muscadine grape extract was effective at reasonably achievable doses, opportunity exists to utilize foods that are readily available and are known to contain resveratrol. This point needs to be viewed with caution until further studies are performed investigating the effects of other resveratrol containing foods like grapes, blueberries, peanuts, or wine. The specific role of resveratrol and the food matrix constituents must be studied to firmly establish that resveratrol is the most important compound present and what role other important compounds play. Third, resveratrol has the potential to reduce radiation-induced aberrations at sites other than the one containing the PU.1 gene providing a wider opportunity for clinical application. This potential would be consistent with our findings that resveratrol and the muscadine grape extract can reduce radiation-induced chromosome aberration frequencies as observed in mouse metaphase bone marrow cells.

Future studies will determine the role of cell cycle arrest and apoptosis in reducing the level of PU.1 gene loss following radiation exposure. In addition, pharmacokinetic studies will examine the concentration of resveratrol found in the bone marrow compartment so that direct comparisons can be made to *in-vitro* studies.

Investigation of piceatannol and pterstilbene as single agents or combined with resveratrol will clarify the potential synergistic role of these grape constituents in reducing the chromosome damage produced by radiation exposure. Our resveratrol results will be used to develop studies investigating the effect of resveratrol and other polyphenolic compounds on the incidence of AML in irradiated mice. These results could set the stage for future clinical investigations and applications.

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- Chapter 5 -

Low Dose Resveratrol and Muscadine Grape Containing Resveratrol Reduce the Frequency of Radiation-induced Bone Marrow Cell Chromosome Aberrations

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Abstract

Resveratrol is a compound that has received a significant amount of attention because of its ability to act as an antioxidant and to induce antioxidant enzymes; reduce inflammatory mediators; and modulate cell cycle progression, signal transduction, and apoptosis. We have previously shown that resveratrol reduces the frequency of radiation-induced chromosome aberrations in metaphase bone marrow cells at a dose of 100 mg/kg. This resveratrol dose, as well as many concentrations used in other reported studies, is not practical for clinical use; therefore, it is important to explore the potential to use doses of resveratrol that are practical. In addition, making clear comparisons between resveratrol as a single agent and resveratrol as found in the food matrix can further delineate important relationships with other food constituents. For this study CBA/CaJ mice were divided into 2 arms for development of a dose response curve for 1) resveratrol as a single agent and 2) muscadine grape extract containing resveratrol. Control groups consisted of mice receiving 1) no treatment, 2) resveratrol only, 3) muscadine grape extract only, and 3) radiation only. Metaphase bone marrow cells were examined on day 1 post-irradiation and the frequency of chromosome aberrations determined. These results make it clear that 100 mg/kg of resveratrol is not the optimum dose of resveratrol as a single agent. Further, a 5.73 μg/kg dose of resveratrol as found in the muscadine grape extract is significantly ($p<0.0001$) better than the same dose of resveratrol (5.75 μg/kg) as a single agent at reducing the frequency of radiation-induced chromosome aberrations in metaphase bone marrow cells. This study provides clear evidence that effective doses of resveratrol can be delivered in physiological doses

found in food and expands the opportunity for widespread use of foods containing resveratrol.

Introduction

Resveratrol (*trans*-3,5,4'-trihydroxystilbene) has been shown to induce cell cycle arrest and apoptosis; alter signal transduction; act as an antioxidant and induce antioxidant enzymes; provide anti-inflammatory effects; and influence DNA repair (1-4). Importantly, resveratrol is found in a large number of plants including grapes, peanuts, blueberries, bilberries, cranberries, lingonberries, and the weed *Polygonum cuspidatum* (5-9). Concentrations of resveratrol in plants vary depending on the plant variety, climatic conditions, and other plant stresses and concentrations have been reported to range from 0.1-14.3 mg/L in red wine, 0.16-3.54 μg/g in grapes, 24.06 μg/g (mean) dry grape skins, and 0.02-1.92 μg/g in peanuts (5). With its wide range of identified molecular targets and food sources, resveratrol has potential for clinical application for a large number of human conditions including cancer, cardiovascular problems, chronic inflammatory conditions, and aging (10).

It is important to recognize that the molecular effects of resveratrol are concentration dependent and are cell line dependent. For example, resveratrol inhibited proliferation of 4 leukemia cell lines (mouse 32Dp210 and L1210; and human U937 and HL-60) with an IC_{50} for each cell line of 18, 20, 26, and 34 μM respectively. These cancer cells are more sensitive to the effects of resveratrol than normal bone marrow progenitor cells that were found to have an IC_{50} of 59 μM. Further, long-term resveratrol (80 μM) exposure reversibly inhibited clonal growth of normal hematopoietic progenitor

cells but at a higher IC_{50} compared to the leukemia cell lines (11). Another study showed the IC_{50} for resveratrol-induced apoptosis in the human leukemia cell lines THP1, HL60, U937, and WSU-CLL (5.07, 15.72, 16.09, and 42.76 μM) varied by cell line and that the IC_{50} for normal human bone marrow cells (60 μM) was higher (12).

Our previous study reported that resveratrol at 100 mg/kg was able to reduce the frequency of radiation-induced bone marrow cell chromosome aberrations observed in mouse metaphase cells (13). This property of resveratrol is important because ionizing radiation is known to create DNA damage that can lead to mutagenesis and carcinogenesis depending on the total dose, dose rate of delivery, and animal species (14-16). Radiation exposure can occur from both occupational and therapeutic sources. In addition, increasing survival following radiation therapy for cancer has led to increased risk of therapy-induced secondary cancers including acute myeloid leukemia (17,18).

The resveratrol dose used in our previous study and doses reported in other *in-vivo* studies are unfortunately too high to be reasonably achieved using wine, grapes, peanuts, or other foods as a source of resveratrol. These studies used doses that ranged from 2.5 mg/kg to 1000 mg/kg of resveratrol orally or by i.p. injection in mouse or rat models (10). Doses in the 10-20 mg/kg range were commonly used and would require 48.95 L of wine with 14.3 mg/L resveratrol or 435 lbs of grapes with 3.54 μg/g resveratrol per day for a 70 kg person. Using a food source for resveratrol delivery is desirable if a food concentration dose of resveratrol can be shown to be effective because food could provide an economical and readily available source of resveratrol. Therefore, it is important to determine if lower doses of resveratrol can be effective *in-vivo* at reducing the radiation-induced chromosome aberration frequencies.

The doses established for resveratrol as a single agent may not be directly comparable to the doses of resveratrol present in foods because foods like grapes are known to contain over 1600 constituents (19). Certain of these constituents may work together to promote beneficial effects even in the low concentrations present. A number of authorities have postulated that low dose combination therapy strategies can be more effective for cancer prevention than those using individual agents (20-21). Combination therapies have been shown to be more effective than single agent therapies for many clinical problems including cancer therapy and pain management. This combination therapy approach has been investigated using the $APC^{Min/+}$ mouse, a model of human familial adenomatous polyposis. These mice were treated with a combination of sulindac (a nonspecific nonsteroidal anti-inflammatory) and EKI-569 (an irreversible inhibitor of the epidermal factor receptor kinase) and significant protection against intestinal tumorigenesis was found even though the sulindac dose used was ineffective as a single agent (22). A recent randomized placebo-controlled, double-blind trial investigating the recurrence of colorectal adenomas in human patients using low doses of difluoromethylorinithine (a polyamine synthesis inhibitor) and sulindac also demonstrated a significant decrease (70%) in recurrence in all adenomas (23).

In the study reported here we use the CBA mouse to investigate the ability of resveratrol as a single agent and a muscadine grape extract with a known *trans*-resveratrol content to reduce the frequency of radiation-induced bone marrow cell chromosome aberrations observed in mouse metaphase cells. Identification of the optimal dose of resveratrol as a single agent is important for future *in-vivo* research exploring the mechanisms of resveratrol's radioprotection. Further, in a like fashion,

determination of the optimal dose of *trans*-resveratrol in the muscadine grape extract matrix is essential. Clearly, the effects of food sources of resveratrol are not directly comparable to the effects of resveratrol as a single agent; therefore, comparison of dose response curves clarifies the extrapolation of results from studies using resveratrol as a single agent to the potential to use food sources as an effective vehicle for resveratrol delivery and food as a therapeutic approach.

Materials and Methods

Animals

Eight to nine week old, male CBA/CaJ mice were obtained from Jackson Laboratory for use in this study. These mice were allowed to acclimate for approximately 1 week prior to use. They were housed and maintained according to the approved standards in the Laboratory Animal Resources facility at Colorado State University (CSU). The Animal Care and Use Committee at CSU approved animal handling and experiments.

Radiation

Mice were placed as a group of 5 into a well ventilated plexiglass container for radiation exposure. γ-radiation was delivered using a ^{137}Cs irradiator – J.L. Shepherd model 81-14. Radiation was delivered to each mouse at a dose rate of 1.13 Gy/min to a total dose of 3 Gy based on dosimetry.

Resveratrol and Muscadine Grape Extract Dosing and Administration

Trans-resveratrol (Sigma-Aldrich) as a single agent was administered in the following doses to groups of mice: 100, 50, 25, 12.5, 6.25, 3.12, and 1.5 mg/kg, and 5.75

μg/kg. The *trans*-resveratrol was dissolved in 100% ethanol to a concentration of 50 mg/ml for a stock solution. Prior to gavage administration, the specified amount of resveratrol stock was further diluted with 100% ethanol to a volume of 0.05 ml and then mixed with distilled water (0.20 ml) for a final gavage volume of 0.25 ml.

Muscadine grape powder (Muscadine Naturals, Inc., Lewisville, NC) was used to create an ethanol extract with a defined concentration of total *trans*-resveratrol. The muscadine grape extract was administered to deliver the following doses of total *trans*-resveratrol to each group of mice: 10.70, 7.13, 5.73, 2.10, and 0.90 μg/kg. Prior to gavage administration, the specified amount of muscadine grape extract stock was removed from the appropriate stock solution, diluted with 100% ethanol, and then mixed with distilled water to provide the indicated dose of total *trans*-resveratrol. The final gavage volume was 0.25 ml: 0.05 ml ethanol/muscadine grape extract stock and 0.20 ml distilled water.

All mice were treated individually with the designated resveratrol as a single agent or muscadine grape extract by gavage every 24 hours for 2 days prior to the day of irradiation and on the day of irradiation, 30 min before radiation exposure.

Muscadine Grape Extract Preparation and Analysis

The muscadine grape extract was prepared by adding 1, 2, 3, or 4 g of muscadine grape powder to 10 ml of 100% ethanol, covered with aluminum foil, and repeatedly shaken. Approximately 24 hours later, suspended material and fluid was collected using a 22 ga needle. Material that would not pass through the 22 ga needle was discarded. The collected suspension placed at -20°C until used for analysis of designated

constituents or administered to the indicated group. This muscadine grape extract stock was used within 48 hours of preparation.

Nine muscadine grape extract stock samples of each concentration (1, 2, 3, and 4 g) were analyzed using tandem mass spectrometry (MDS Sciex 3200 Q TRAP®, MDS Analytical Technologies, Concord, Ontario, Canada) coupled with high-performance liquid chromatography (Agilent 1200 Series Binary Pump SL, Foster City, CA). Each muscadine grape extract stock was diluted 1:5 in methanol, an internal standard was added (up to 100 ng/ml), the solution was filtered with a 2 μm filter, and then injected in the liquid chromatography/mass spectrometer. Quantitative analysis for the following compounds was performed: *trans*-resveratrol, *trans*-piceid (*trans*-resveratrol-3-O-D-glucoside), *trans*-piceatannol (*trans*-3,4,3',5'-tetrahydroxystilbene), and *trans*-pterostilbene (3,5-dimethoxy-4'-hydroxystilbene). Standards for *trans*-resveratrol, *trans*-piceid, *trans*-picetannol, and *trans*-ptersotilbene were obtained from Sigma-Aldrich and the astringin standard was obtained from Sequoia Research Products (Pangbourne, UK). Following quantitative analysis for these compounds, the mean ± SD were derived. In addition, where appropriate the related aglycone and glycoside forms were totaled and the mean ± SD was derived.

Bone Marrow Collection and Preparation

Colchicine (0.1 ml of 0.5%), a mitotic blocker, was administered i.p. to each mouse one hour prior to bone marrow collection. Bone marrow was flushed from each femur, tibia, and radius with PBS and placed into PBS. The PBS bone marrow mixture was centrifuged at 1000 rpm for 5 min at 4°C. The cell concentrate was then placed into an incubation mixture (9 ml 0.75 M KCl, 1 ml EDTA trypsin, 200 μl colcemid), mixed,

and incubated for 30 min at 37°C. Following incubation, 5 ml fresh fixative (3:1 methanol:acetic acid) was added, mixed, and centrifuged at 1000 rpm for 5 min. Cells were washed with fresh fixative 2 more times. The supernatant was decanted and a small amount of fresh fixative was added and cells were dropped onto cleaned microscope slides.

Chromosome Aberration Scoring

Slides with bone marrow metaphase cells were stained with 10% Giemsa stain for 7 min, rinsed, air dried, and mounted with a glass coverslip. The slides were coded, blinded, and 25 metaphases with 38-40 chromosomes each were scored for aberrations. Chromosome aberrations observed in metaphase bone marrow cells were scored as follows: 1) fragments, identified as un-rejoined chromosome fragments, 2) gaps, identified as a separation along the length of a chromatid that was less than the width of the chromatid, 3) dicentrics, identified as a chromosome (or chromatid) end joined to another chromosome (or chromatid) along with any associated compound rejoined fragments, and 4) Robertsonian translocations, identified as two chromosomes joined at their centromeres. After scoring, the slides were unblinded and results were compiled according to the respective treatment group. Chromosome aberrations of all types were combined to give a total that was used for statistical calculations.

Effect of Resveratrol or Muscadine Grape Extract on Radiation-induced Bone Marrow Cell Chromosome Damage

A total of 160 9-10 week old, male CBA/CaJ mice (24.7±0.7 g) were divided into groups of 10 mice each as follows: 1) no treatment (No Tx), 2) resveratrol (RES) only (100 mg/kg), 3) muscadine grape extract (MGE) only (5.73 μg/kg) 4) radiation (RAD)

only (3-Gy), 5) Res+RAD dose groups (100, 50, 25, 6.25, 3.12, 1.50 mg/kg and 5.75 μg/kg resveratrol as a single agent followed by 3 Gy radiation), or 6) MGE+RAD dose groups (10.70, 7.13, 5.73, 2.10, and 0.90 μg/kg total *trans*-resveratrol followed by 3 Gy radiation). Note that results from the control groups (No Tx, RES, and RAD) and 1 treatment group (Res+RAD 100 mg/kg) were previously reported (13).

One day following whole body irradiation, bone marrow from each mouse was individually harvested, processed, slides prepared and metaphase chromosome aberrations scored as described above.

Statistical Analysis

Statistical analysis consisted of evaluation of mean total bone marrow cell chromosome aberrations observed in each treatment group. Total aberrations represent the sum of all chromosome aberration types observed. The data are presented as mean total $\pm$ standard deviation chromosome aberrations per metaphase bone marrow cell. Since the data exhibited non-normality and heteroscedasticity, the non-parametric Kruskal-Wallis ANOVA was used for overall comparisons between the treatment groups and the Wilcoxon rank-sum tests were used to compare total chromosome aberration frequencies between pairs of groups.

Results

Resveratrol was able to significantly reduce the frequency of observed radiation-induced mean total chromosome aberration frequency in mouse metaphase bone marrow cells at 1 day following irradiation in all doses of resveratrol (5.75 μg/kg and 1.50 mg/kg-

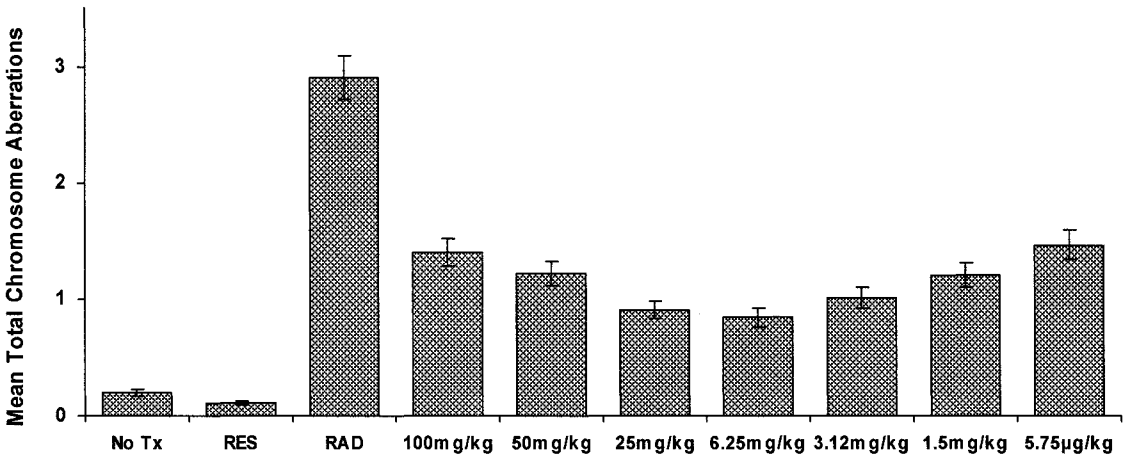

Figure 1. Dose response curve based on the mean total chromosome aberration frequency observed in metaphase mouse bone marrow cells on day 1 post-irradiation. Results of 250 bone marrow metaphase cells per group: no treatment (No Tx), resveratrol (RES) only, radiation (RAD) only, and each resveratrol plus radiation dose group using resveratrol as a single agent at the indicated doses are presented.

100 mg/kg) compared to the RAD only group (Table 1 and Figure 1). The overall Kruskal Wallis ANOVA for the mean total chromosome aberrations was p=0.000.1. The optimal dose of resveratrol as a single agent, based on the observed mean total chromosome aberration frequency at 1 day post-irradiation was 6.25 mg/kg (0.85 ± 1.22) (Table 1). However, this mean total chromosome aberration frequency was not statistically different than those seen for 25 mg/kg (0.92 ± 1.29) or 3.12 mg/kg (1.02 ± 1.36) with p-value of 0.545 and 0.185 respectively. This indicates that a dose range of 3.12-25 mg/kg results in similar reductions in mean total chromosome aberration frequencies (Tables 1 and 2).

Interestingly, the 2 resveratrol doses greater (100 and 50 mg/kg) than 25 mg/kg had higher mean total chromosome aberration frequencies. Doses of 100 mg/kg (1.40 ± 1.92) and 50 mg/kg (1.22 ± 1.59) resulted in statistically higher mean total chromosome

TABLE 1

Mean Chromosome Aberrations per Bone Marrow Metaphase Cell Observed in Each Mouse Group with Each Treatment

	Total	Fragments	Gaps	Dicentrics	Robertsonian
No treatment					
	0.20 ± 0.55	0.06 ± 0.28	0.07 ± 0.38	0.01 ± 0.11	0.06 ± 0.25
Resveratrol					
	0.12 ± 0.39	0.02 ± 0.18	0.04 ± 0.26	0.02 ± 0.13	0.03 ±0.18
Muscadine Grape Extract					
	0.20 ± 0.41	0.03 ± 0.17	0.06 ± 0.25	0.02 ± 0.14	0.09 ± 0.28
Radiation					
	2.90 ± 3.04	2.12 ± 2.65	0.33 ± 0.70	0.28 ±0.28	0.17 ±0.50
Resveratrol + radiation					
100 mg/kg	1.40 ± 1.92	0.88 ± 1.67	0.22 ± 0.60	0.19 ± 0.49	0.12 ± 0.34
50 mg/kg	1.22 ± 1.59	0.92 ± 1.46	0.18 ± 0.46	0.02 ± 0.15	0.10 ± 0.31
25 mg/kg	0.92 ± 1.29	0.61 ± 1.17	0.20 ± 0.47	0.02 ± 0.14	0.09 ± 0.30
6.25 mg/kg	0.85 ± 1.22	0.56 ± 1.10	0.15 ± 0.42	0.02 ± 0.14	0.11 ± 0.35
3.12 mg/kg	1.02 ± 1.36	0.75 ± 1.24	0.12 ± 0.37	0.03 ± 0.17	0.12 ± 0.34
1.5 mg/kg	1.21 ± 1.51	0.72 ± 1.38	0.06 ± 0.25	0.10 ± 0.31	0.34 ± 0.58
5.75μg/kg	1.47 ± 1.84	1.15 ± 1.78	0.19 ± 0.50	0.02 ± 0.15	0.10 ± 0.31
Muscadine Grape Extract + radiation					
10.70 μg/kg	1.19 ± 1.69	0.95 ± 1.65	0.11 ± 0.34	0.04 ± 0.19	0.09 ± 0.30
7.13 μg/kg	0.76 ± 1.24	0.57 ± 1.17	0.10 ± 0.35	0.01 ± 0.11	0.08 ± 0.29
5.73 μg/kg	0.52 ± 0.91	0.33 ± 0.82	0.04 ± 0.19	0.02 ± 0.18	0.13 ± 0.34
2.10 μg/kg	0.52 ± 0.82	0.30 ± 0.72	0.05 ± 0.23	0.04 ± 0.21	0.13 ± 0.36
0.90 μg/kg	1.08 ± 1.49	0.80 ± 1.42	0.09 ± 0.34	0.03 ± 0.19	0.15 ± 0.39

Note. Values are means ± SD based on 250 cells counted for each treatment group on day 1 post-irradiation. The doses listed for the muscadine grape extract are for the total *trans*-resveratrol administered. Radiation was 3 Gy whole-body γ-radiation. Total includes fragments, gaps, dicentrics, and Robertsonian translocations. Fragments include chromosome and chromatid types, including interstitial deletions, terminal deletions, and breaks. Gaps include chromatid and isochromatid types. Dicentrics were of the chromatid type and Robertsonian translocations were of the chromosome type.

aberration frequencies when compared to the 25 mg/kg dose while doses less than 3.12 mg/kg also resulted in higher mean total chromosome aberration frequencies: 1.50 mg/kg (1.21 ± 1.51) and 5.75 μg/kg (1.47 ± 1.84). Surprisingly, the mean total chromosome aberration frequency observed following the single agent resveratrol dose of 5.75 μg/kg, was not statistically different than the 100 mg/kg dose with a p-value of 0.586 indicating that the highest dose (100 mg/kg) of resveratrol was not more effective than the lowest resveratrol dose (5.75 μg/kg).

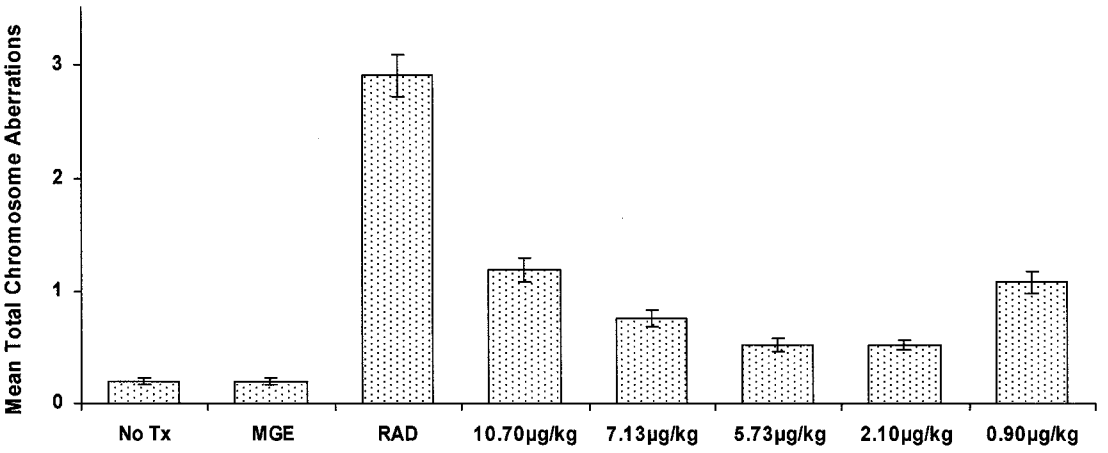

Figure 2. Dose response curve based on the mean total chromosome aberration frequency observed in metaphase mouse bone marrow cells on day 1 post-irradiation for the muscadine grape extract groups. There 250 metaphase bone marrow cells scored in each group. No Tx – no treatment, MGE – muscadine grape extract at 5.73 µg/kg, RAD – radiation only at 3 Gy, and the doses indicate the total *trans*-resveratrol amount administered to each mouse before radiation as described.

In general terms, as the resveratrol dose decreases to 3.12 mg/kg in the Res+RAD groups, the fragments, gaps, and dicentrics decrease in frequency relative to the results for 100 mg/kg Res+RAD group while the Robertsonian translocations are similar. The 5.75 µg/kg Res+RAD dose group had an increase in fragments relative to the 100 mg/kg Res+RAD group; however, the gaps and Robertsonian translocations were similar to the 100 mg/kg Res+RAD group. Additionally, there was an increase in the fragments, a minor increase in gaps, and no change in dicentrics and Roberstonian translocations when the 5.75 µg/kg Res+RAD group was compared to the 3.12 mg/kg Res+RAD group.

The microgram per kilogram doses of *trans*-resveratrol found in the muscadine grape extract was also able to reduce the radiation-induced mean total chromosome aberration frequency (Table 1 and Figure 2). The overall Kruskall Wallis ANOVA for the mean total chromosome aberration frequency was $p<0.0001$. Consistent with the dose

TABLE 2
Wilcoxon-rank Sum Test p-values for Pair-wise Comparisons of Mean Chromosome Aberration Frequency for Resveratrol as a Single Agent in each Treatment Groups

	100 mg/kg	50 mg/kg	1.5 mg/kg	3.12 mg/kg	25 mg/kg	6.25 mg/kg
5.75 μg/kg	0.586	0.217	0.386	0.011	0.001	0.0001
100 mg/kg		0.500	0.784	0.046	0.007	0.001
50 mg/kg			0.651	0.173	0.034	0.007
1.5 mg/kg				0.062	0.007	0.001
3.12 mg/kg					0.460	0.185
25 mg/kg						0.545

Note. Dose indicates amount of resveratrol administered as a single agent.

response pattern found with resveratrol as a single agent, the doses of *trans*-resveratrol in the muscadine grape extract with the lowest mean total chromosome aberration frequency were 2.10 μg/kg (0.52 ± 0.82) and 5.73 μg/kg (0.52 ± 0.91) in these MGE+RAD groups (Table 1). These results were not significantly different than the MGE+RAD 7.13 μg/kg (0.76 ± 1.24) with p-values of 0.113 when compared to MGE+RAD 2.10 μg/kg and 0.063 when compared to MGE+RAD 5.73 μg/kg (Table 3). However, MGE+RAD 7.13 μg/kg was significantly different than MGE+RAD 10.70 μg/kg (1.19 ± 1.69) and the MGE+RAD 2.10 μg/kg dose was significantly different than MGE+RAD 0.90 μg/kg (1.08 ± 1.49). Based on these results comparing the mean total chromosome aberration frequencies, the optimum dose range of *trans*-resveratrol present in the muscadine grape extract is 2.10-7.13 μg/kg.

In general terms, as the *trans*-resveratrol dose from the muscadine grape extract is decreased from 10.70 μg/kg to 2.10 μg/kg in the MGE+RAD groups, the frequency of fragments and gaps decreases while the dicentrics and Robertsonian translocations are similar. For the MGE+RAD 0.90 μg/kg group the fragments increased compared to the

MGE+RAD 2.10-7.13 μg/kg dose groups. The gaps and dicentrics were similar to the MGE+RAD 10.70 μg/kg group.

TABLE 3
Wilcoxon-rank Sum Test p-values for Pair-wise Comparisons of Mean Chromosome Aberration Frequency for Muscadine Grape Extract Treatment Groups

	MGE				RES
MGE	**7.13 μg/kg**	**5.73 μg/kg**	**2.10 μg/kg**	**0.90 μg/kg**	**5.75 μg/kg**
10.70 μg/kg	0.006	<0.0001	<0.0001	0.801	0.047
7.13 μg/kg		0.063	0.113	0.009	<0.0001
5.73 μg/kg			0.756	<0.0001	<0.0001
2.10 μg/kg				<0.0001	<0.0001
0.90 μg/kg					0.021

Note. MGE indicates total *trans*-resveratrol as found in muscadine grape extract and RES indicates *trans*-resveratrol as a single agent.

When comparing the reductions in radiation-induced mean total chromosome aberrations between mg/kg doses of resveratrol as a single agent and the μg/kg doses of *trans*-resveratrol in muscadine grape extract, it can be seen that the muscadine grape extract reduced the mean total chromosome aberration frequency to a lower level than the significantly higher doses of resveratrol alone. In addition, equivalent doses of resveratrol alone (5.75 μg/kg) and *trans*-resveratrol in the muscadine grape extract (5.73 μg/kg) were directly compared and found to achieve significantly different reductions in mean total chromosome aberration frequency with $p<0.0001$. This demonstrates that the muscadine extract is superior to resveratrol as a single agent.

The total *trans*-resveratrol content, representing the combination of the aglycone and glycoside forms of *trans*-resveratrol, of the muscadine grape extract found in each muscadine grape concentration (MGE-1g, MGE-2g, MGE-3g, and MGE-4g) increased with increasing amounts of muscadine grape powder added to the ethanol (Table 4 and

Figure 3). It is important to note that *trans*-piceatannol (*trans*-3,4,3',5'-tetrahydroxystilbene), and *trans*-pterostilbene (3,5-dimethoxy-4'-hydroxystilbene) were also identified in the muscadine grape extract. The aglycone content of these additional polyphenolic stilbenes is shown in Table 5 and Figure 4.

TABLE 4
Total *trans*-resveratrol Measured in Each Muscadine Grape Extract in ng/ml

	MGE-1g	MGE-2g	MGE-3g	MGE-4g
Mean	750.06±74.69	1340.14±146.05	1790.46±262.46	2229.44±179.49

Note. Total *trans*-resveratrol ± SD in ng/ml present in the muscadine grape extract for each concentration of muscadine grape powder used in this study. Nine samples of each muscadine grape extract concentration were analyzed for *trans*-resveratrol and *trans*-piceid content. The combination of *trans*-resveratrol and *trans*-piceid gives the total *trans*-resveratrol indicated in this table.

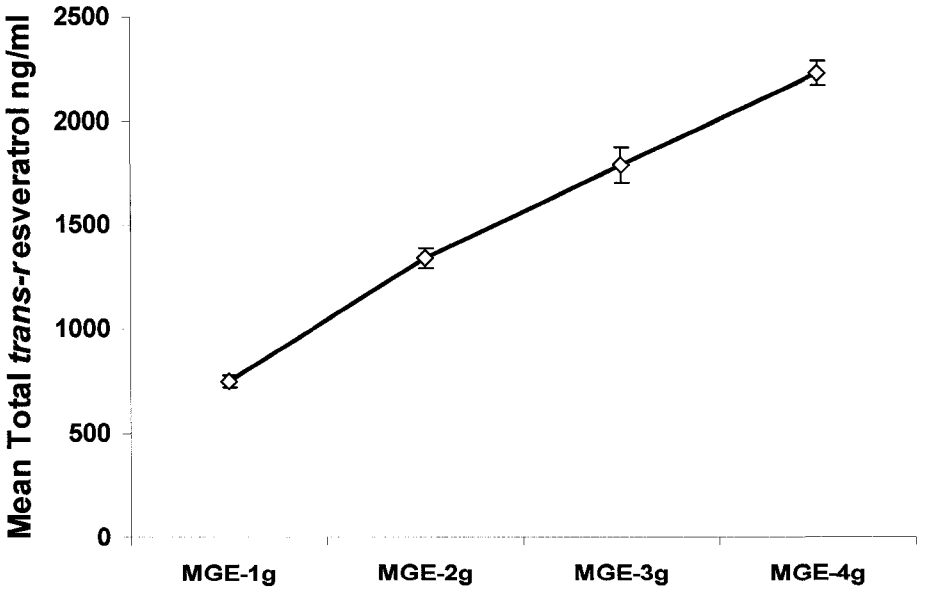

Figure 3. Muscadine grape extract (MGE) analysis for each amount of muscadine grape powder added (1, 2, 3, or 4 g) to 10 ml of 100% ethanol for the total amount of *trans*-resveratrol ± SE in ng/ml. Total *trans*-resveratrol is the combined amount of the aglycone and glycoside forms of *trans*-resveratrol present in the muscadine grape extract. Nine samples of each extract concentration were analyzed for each data point.

TABLE 5
Muscadine Grape Extract Select *trans*- Stilbene Aglycone Content in ng/ml

	MGE-1g	MGE-2g	MGE-3g	MGE-4g
Piceatannol	108.09±20.59	268.61±85.43	472.89±84.27	725.87±93.45
Pterostilbene	386.19±67.71	750.79±101.85	1109.24±40.32	1364.14±156.09
Resveratrol	497.77±69.84	968.15±77.26	1277.48±245.14	1605.11±196.73

Note. Nine samples of each muscadine grape extract (MGE-1g, MGE -2g, MGE-3g, and MGE-4g) concentration were analyzed for *trans*- resveratrol, *trans*-piceatannol, and pterostilbene aglycones. The results are reported in ng/ml ± SD.

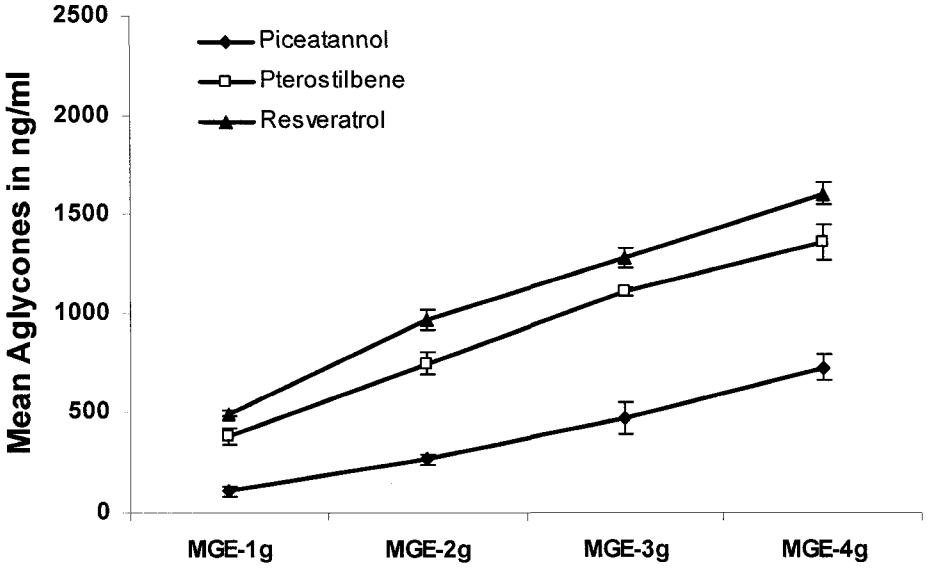

Figure 4. Muscadine grape extract analysis of 9 samples for each data point indicates the amounts of the aglycone forms of selected polyphenolic stilbenes – *trans*-resveratrol, *trans*-piceatannol, and pterostilbene. Results are in ng/ml ± SE.

Discussion

This study comparing resveratrol as a single agent to a muscadine grape extract administered based on μg/kg dosing of the *trans*-resveratrol content shows that the

muscadine grape extract is significantly better at reducing the frequency of radiation-induced chromosome aberrations in mouse metaphase bone marrow cells. Further, this study demonstrated that the highest doses of resveratrol as a single agent or as administered in the muscadine grape extract do not provide the optimum results. These 2 observations are important because the μg/kg doses found in food have been considered too low to have meaningful cell and molecular effects *in-vivo*. However, it is important to note that 5.75 μg/kg of resveratrol as a single agent had equivalent reductions in mean total chromosome aberrations as the 100 mg/kg (1.47 ± 1.84 and1.40 ± 1.92 respectively); but, the 5.73 μg/kg dose of *trans*-resveratrol in the muscadine grape extract was significantly better than the 5.75 μg/kg dose of resveratrol alone (0.52 ± 0.91 and 1.47 ± 1.84 respectively).

These findings indicate that food sources of resveratrol, such as the grape, can provide quantities of resveratrol that are sufficient to effect molecular changes that reduce radiation damage to chromosomes as observed in the mouse bone marrow cells. In addition, the delivery of resveratrol in conjunction with other small molecules found in food, has an effect that is superior to resveratrol alone at the same dose ($p<0.0001$). While the results of the muscadine grape extract are consistent with those observed with resveratrol alone in the same model this study does not conclusively show that the *trans*-resveratrol in the muscadine grape extract is the small molecule responsible for the observed reduction in radiation-induced chromosome aberrations, it does show that providing a quantity of muscadine grape extract based on the *trans*-resveratrol content is sufficient to have an effect.

With this in mind it is important to explore the potential for other muscadine grape extract constituents to mediate a molecular effect either alone or in conjunction with other muscadine grape constituents. We were able to measure *trans*-piceatannol and *trans*-pterostilbene, in addition to the *trans*-resveratrol, in the muscadine grape extract. These compounds were selected as a focus because they are structurally similar to *trans*-resveratrol and have been shown to have similar molecular effects to *trans*-resveratrol. The ability of the muscadine grape extract, with its μg/kg dose of *trans*-resveratrol, to reduce the radiation-induced frequency of chromosome aberrations in mouse bone marrow cells seen in this study is likely due to the combined effects of these structurally related stilbenes.

Trans-piceatannol has been shown to inhibit tyrosine kinase activity involved in cell proliferation, tubulin phosphorylation, and phosphorylation of DNA transcription factors (24). Further, piceatannol was found to induce apoptosis in lymphoma cells BJAB through caspase-3 activation and was a more effective inducer of apoptosis in primary leukemic lymphoblasts than resveratrol in a concentration dependent manner (25). In the leukemic cell line KBM-5, piceatannol inhibited TNF-mediated NF-κB activation in a concentration dependent fashion (26); induced apoptosis in the leukemia cell line U937 by down regulating Bcl-2 and activating caspase-3 (27); and induced apoptosis in leukemia cell lines HL60, Hl60R, K562, and HUT78 and increased cell accumulation in S phase in HL60 and K562 cells (28). In addition to piceatannol being present in the muscadine grape extract, resveratrol is metabolized to piceatannol by the cytochrome P450 enzymes CYP1B1 and CYP1A2 (24,29).

Trans-ptersotilbene has also been shown to have similar properties as resveratrol, acting as an antioxidant (30); moderately inhibiting COX-1 and weakly inhibiting COX-2 (30); inducing apoptosis in leukemia cell lines (HL60, Hl60R, K562, and HUT78 (28); and cell cycle accumulation in S phase in HL60 and K562 cells (28).

It is also important to consider why the highest doses of resveratrol either alone or in the muscadine grape extract were not the most effective at reducing the chromosome aberrations. This is probably due to a number of different molecular processes that are related to the stilbene exposure time of the affected cells, the tissue concentrations achieved with the doses used, the overall potential differential sensitivity of the bone marrow cell lines and their stage of maturation to the effects of the stilbenes, and the normal cell kinetics. These are important considerations because the molecular effects of resveratrol, piceatannol, and pterostilbene have been shown to be concentration dependent and cell line dependent (28,31). As already discussed, normal cells are less sensitive to the effects of resveratrol than the immortalized cancer cell lines. As a result of this combination of factors, it is likely that at the 100 mg/kg resveratrol dose (Res+RAD) there is a partial cell cycle arrest in early S phase. As the resveratrol dose decreases into the 3.12-25 mg/kg (Res+RAD) range the cell cycle arrest is less prominent. Cells in early S phase have an intermediate sensitivity to radiation damage and would be expected to have an increased frequency of chromosome aberrations with irradiation. This increased early S phase arrest and resultant increased frequency of bone marrow cell chromosome aberrations can be seen in the increased micronuclei formation seen in irradiated mouse bone marrow cells exposed to 100 mg/kg resveratrol alone (Res+RAD) daily for 2 days before radiation exposure compared to the frequency seen in

the radiation only cells (see Chapter 3). Interestingly, the increase in chromosome damage in cells receiving both resveratrol and radiation, as shown with the micronuclei assay, does not result in an increase in mean total chromosome aberrations seen in metaphase bone marrow cells for 2 main reasons. First, since the bone marrow cells have undergone a partial S phase arrest, fewer cells are undergoing mitosis and therefore fewer damaged cells are being counted. Second, damaged cells are undergoing apoptosis and therefore, not progressing into mitosis. When considering the 1.5 mg/kg and 5.75 μg/kg resveratrol alone (Res+RAD) results, the bone marrow cells are likely not arrested in early S phase to the same extent as the 100 mg/kg dose since this arrest is dose dependent. Therefore, these asynchronous bone marrow cells are expected to experience a lower frequency of chromosome damage. As a result, the predominant molecular effect would be DNA repair and apoptosis, depending on the degree of DNA damage, which would reduce the number of damaged cells progressing into mitosis. Since the resveratrol dose is low, resveratrol may not be influencing DNA repair or apoptosis to the same extent as the higher doses.

For the muscadine grape extract at the resveratrol doses used in the MGE+RAD groups, the explanation is less clear even though the dose response curve has the same general shape with the highest (10.70 μg/kg) and lowest (0.90 μg/kg) doses having the greatest mean total chromosome aberration frequency compared to the middle doses (2.10-7.13 μg/kg). However, there are a number of important points to be made. The synergistic effects of the constituents in the muscadine grape extract on specific molecular targets may result in effects that are equivalent to the higher single agent resveratrol effects. In other words the muscadine grape extracted delivered at the dose of

10.70 μg/kg *trans*-resveratrol may facilitate cell cycle arrest and apoptosis to a similar extent as the 100 mg/kg resveratrol dose. The mean total chromosome aberration frequency is not significantly different when comparing the 10.70 μg/kg muscadine grape extract (1.19 ± 1.69) and 100 mg/kg resveratrol (1.40 ± 1.92) dose groups. Also of interest is the observation that a significant difference is not seen when comparing the 0.90 μg/kg muscadine grape extract (1.08 ± 1.49) to the 1.5 mg/kg resveratrol (1.21 ± 1.51) mean total chromosome aberration frequencies in the respective dose groups.

The preceding discussion implies that the same molecular targets are being modulated by both the resveratrol alone and the muscadine grape extract. However, it is feasible that different molecular targets are being modulated by the respective doses of resveratrol alone or in combination with the muscadine grape extract constituents, yielding the same overall cellular responses of cell cycle arrest, DNA repair, and apoptosis. Resveratrol is known to modulate a wide range of molecular targets in a dose and time dependent manner. For example, resveratrol has been shown to induce apoptosis by depletion of inhibitors of apoptosis proteins via p53-independent induction of p21 and a p21-mediated cell cycle arrest associated with survivin depletion (32,33), sensitization of death receptors like TRAIL (TNF-related apoptosis-inducing ligand) (32), down regulation of Bcl-x(L) and Mcl-1 via inhibition of the ERK1/2 pathway (34), depolarization of the mitochondria membrane potential and activation of caspase-9 (35), activation of caspase-2 (36), and caspase-3 activation (37).

Future investigations will define the respective cellular mechanism and protein targets that underlie the observations reported here associated with the ability of resveratrol alone or in the muscadine grape extract to reduce the radiation-induced

chromosome aberration frequency. Until the mechanism is determined, the important point is that resveratrol alone is capable of reducing chromosome aberrations, that lower doses of resveratrol provide superior effects in regard to the reduction in chromosome aberrations, and that resveratrol combined with other muscadine grape extract constituents is markedly better at microgram doses of resveratrol than the mg/kg doses of resveratrol alone or at comparable doses (5.75 μg/kg).

The study reported here did not conclusively determine that resveratrol was the constituent that reduced the radiation-induced chromosome aberrations. However, it did clearly demonstrate that the microgram/kg doses of the muscadine grape extract constituents is superior to the mg/kg dose of resveratrol as a single agent at reducing the frequency of observed chromosome aberrations in irradiated mouse bone marrow cells 1 day after 3 Gy whole body γ-radiation. These results are significant because one of the barriers to widespread use of plant based constituents has been limited by the amounts present in foods or other plant sources. In addition, with the reduction in chromosome aberrations shown in this study, a reduction in later cancer risk would logically result giving muscadine grape significant potential for clinical application once a clear understanding of the molecular mechanism has been defined.

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- Chapter 6 -

Discussion

The series of experiments reported here had a number of aims including 1) determination if resveratrol could reduce radiation-induced chromosome aberrations in mouse bone marrow cells, 2) determination if resveratrol could reduce the radiation-induced loss of the PU.1 gene in mouse bone marrow cells, 3) determination of the dose of resveratrol that optimally reduces radiation-induced chromosome aberrations in mouse bone marrow cells, and 4) determination if a food source of resveratrol like muscadine grape is capable of reducing the frequency of chromosome aberrations and PU.1 gene loss in irradiated bone marrow cells. These aims were fulfilled using the CBA mouse, which develops acute myeloid leukemia (AML) following irradiation, resveratrol, and a muscadine grape extract. Experiments were arranged so that mice were divided into the following control and treatment groups: 1) no treatment (No Tx), 2) resveratrol (100 mg/kg) only (RES), 3) radiation (3 Gy γ-radiation) only (RAD), 4) resveratrol initiated before radiation exposure (Res+RAD), 5) resveratrol started after irradiation (RAD>Res), and 6) muscadine grape extract administered before radiation exposure. Bone marrow was harvested on days 1, 7, or 30 following irradiation or at an equivalent end point.

For the cytogentic studies, 10 mice were include in each group with a total of 250 cells scored for chromosome aberrations; the PU.1 gene loss studies included 5 mice in each group with a total of 500 cells scored in each group. Chromosome aberration categories included fragments, gaps, dicentrics, Robertsonian translocations, and the total observed in bone marrow metaphase cells. Means ± standard deviations were developed for each category. These values were used for statistical comparison of groups. PU.1 gene loss was performed using a BAC probe for the PU.1 gene. Only metaphase cells

with loss of 2 alleles on sister chromatids were considered to have lost the PU.1 gene. The mean ± standard deviation was used for statistical comparison of groups.

When considering the experiments described in chapter 2, where resveratrol was initiated 2 days before radiation, the no treatment and resveratrol only groups had a low frequency of mean total chromosome aberrations on days 1 (0.20 ± 0.55 and 0.12 ± 0.39 respectively) and 30 (0.14 ± 0.45 and 0.07 ± 0.27 respectively) post-irradiation and the radiation only group had the highest frequency of mean total chromosome aberrations on days 1 (2.90 ± 3.04) and 30 (0.51 ± 0.87). These values compare to those from the resveratrol before radiation group at days 1 (1.40 ± 1.92) and 30 (0.18 ± 0.47). The overall Kruskal-Wallis ANOVA for the mean total chromosome aberrations was $p<0.0001$ for days 1 and 30. Wilcoxon rank-sum tests were used to compare mean total chromosome aberrations between groups. These comparisons showed that there was a statistically significant difference at $p<0.05$ for the following comparisons of mean total chromosome aberrations between groups: 1) no treatment vs. radiation, 2) resveratrol vs. radiation, 3) radiation vs. resveratrol and radiation, and 4) no treatment vs. resveratrol and radiation at day 1 after radiation exposure. For the day 30 end point, a significant difference at the $p<0.05$ was seen for 1) no treatment vs. radiation, 2) resveratrol vs. radiation, and 3) radiation vs. resveratrol and radiation.

In the experiments described in chapter 3, where resveratrol was administered as a single dose 2 hours after radiation exposure (RAD>Res 2 hrs), initiated 2 hours after irradiation and continued to the designated end point (RAD>Res 2 hrs+), or 2 days after radiation exposure and continued (RAD>Res 2 days+), the radiation group had the highest mean total chromosome aberration frequency (0.48 ± 0.76 and 0.51 ± 0.87) at the

7 and 30 day end point. This compares to the results found in the RAD>Res 2 hrs (1.00 ± 1.38, 0.34 ± 0.63 and 0.21 ± 0.44) at 1, 7, and 30 days respectively; RAD>Res 2 hrs+ (0.23 ± 0.47) at 30 days; and RAD>Res 7 days+ (0.30 ± 0.62 and 0.37 ± 0.76) at 7 and 30 days. The Wilcoxon rank-sum tests were used to compare mean total chromosome aberration frequencies. Statistically significant differences at $p<0.05$ were found for the following comparisons: 1) no treatment vs. radiation at 7 and 30 days, 2) radiation vs. RAD>Res 2 hrs at 1, 7, and 30 days, 3) radiation vs. RAD>Res 2 hrs+ at 30 days, and 4) radiation vs. RAD>Res 2 days+ at 7 and 30 days.

Interestingly, administration of resveratrol 2 hours after radiation exposure (RAD>Res 2 hrs) significantly ($p<0.05$) reduced mean total chromosome aberrations more than resveratrol given before radiation (Res+RAD) at 1 day. A significant difference was not seen between RAD>Res 2 hrs and RAD>Res 2 days+ at 7 days and between RAD>Res 2 hrs and RAD>Res 2 hrs+ at 30 days. The RAD>Res 2 hrs, RAD>Res 2 hrs+, and RAD>Res 2 days+ when individually compared to Res+RAD at 30 days gives a $p<0.05$, $p\sim0.05$, and $p>0.05$ respectively.

With these results in mind, a series of micronuclei assay experiments were performed that included the following groups: 1) no treatment, 2) resveratrol (100 mg/kg) only, 3) radiation (3 Gy) only, 4) radiation before resveratrol, and 5) radiation after resveratrol. Bone marrow was collected 24 hours after irradiation and placed into RPMI 1640 media containing 1 μg cytochalasin B per 1 ml for 18 hours. Binucelate cells were examined for the presence of micronuclei. These experiments showed that cells from mice receiving resveratrol 2 hours after irradiation (RAD>Res 2 hrs) had a mean frequency of micronuclei (0.065 ± 0.25) that was similar to the radiation only group

(0.057 ± 0.23) while the group receiving resveratrol before radiation (Res+RAD) had the highest mean frequency of micronuclei (0.093 ± 0.29). Using the Chi-square test to compare groups, statistically significant differences were seen between radiation (RAD) and resveratrol before radiation (Res+RAD) ($p=0.0005$), resveratrol before radiation (Res+RAD) and radiation before resveratrol (RAD>Res) ($p=0.0338$), and radiation (RAD) and radiation before resveratrol (RAD>Res 2 hrs) ($p=0.5746$). The micronuclei indicate that chromosome damage has occurred and these damaged DNA fragments are extruded during cell division. With this in mind it can be seen that there is more DNA damage in cells that have been exposed to resveratrol before radiation (Res+RAD) and there is an equivalent frequency of micronuclei in the radiation (RAD) and radiation before resveratrol (RAD>Res 2 hrs) groups. Yet, the mean total chromosome aberration frequency is lower in the Res+RAD group compared to RAD and in the RAD>Res 2 hrs compared to RAD.

The ability of resveratrol to reduce the frequency of radiation-induced DNA damage was further evaluated using a BAC probe for the PU.1 gene found on chromosome 2 in the mouse. It has been shown that loss of the PU.1 gene occurs following irradiation of CBA mice and that loss of one allele of the PU.1 gene and a missense mutation of the allele on the sister chromatid leads to acute myeloid leukemia (1). As described in chapter 4, mice were divided into the same groups as used for the cytogenetic studies: 1) no treatment, 2) resveratrol only, 3) radiation only, 4) resveratrol before radiation (Res+RAD), and 5) radiation before resveratrol (RAD>Res 2 hrs or RAD>Res 2 days+) with end points at 1 and 30 days after radiation exposure. The mean percent PU.1 loss ± SE was calculated and the Chi-square test was used to calculate

statistical significance. As expected, the radiation only group (11.00 ± 1.40 and 5.60 ± 1.03) had a higher frequency of mean percent PU.1 gene loss than the no treatment group (0.80 ± 0.04 and 1.20 ± 0.49) at 1 and 30 days. When resveratrol was given before radiation (Res+RAD) the mean percent PU.1 loss (1.40 ± 0.53 and 0.40 ± 0.28) was significantly ($p<0.0001$) reduced compared to the radiation only (RAD) groups at 1 and 30 days post-irradiation. Likewise, a significant ($p<0.0001$) reduction in PU.1 gene loss was observed at day 1 and 30 when resveratrol was started 2 hours after radiation (RAD>Res 2 hrs) (1.40 ± 0.50 and 0.80 ± 0.40) compared to the radiation only (RAD) on each day. When resveratrol was initiated 2 days after irradiation and continued (RAD>Res 2 days+) (1.13 ± 0.05) there was a significant ($p=0.0029$) reduction in PU.1 gene loss compared to the radiation only (RAD) group.

The preceding studies were all performed using a 100 mg/kg dose of resveratrol. This dose was empirically chosen and may not be the optimum dose for reducing radiation-induced chromosome aberration frequencies. Therefore a series of mouse experiments, as described in chapter 5, was performed using deescalating doses of resveratrol as follows: 50, 25, 6.25, 3.12, and 1.50 mg/kg. These doses were administered by gavage every 24 hrs for 3 days with the third dose given 30 minutes before radiation exposure. Bone marrow was collected on day 1 following irradiation and processed for cytogenetic evaluation. Previously mentioned control (No Tx, RES, and RAD) and the 100 mg/kg resveratrol before radiation (Res+RAD) groups were included as part of the development of this dose response curve. The mean total chromosome aberration frequency was determined and the overall Kruskall Wallis ANOVA and the Wilcoxon rank-sum tests were used for comparisons. Overall ANOVA

was p=0.0001. All resveratrol and radiation dose groups had a decrease in radiation-induced mean total chromosome aberration frequencies: 100 (1.40 ± 1.92), 50 (1.22 ± 1.59), 25 (0.92 ± 1.29), 6.25 (0.85 ± 1.22), 3.12 (1.02 ± 1.36), and 1.5 mg/kg (1.21 ± 1.51). Based on these results and statistical comparisons the optimum resveratrol dose for reducing radiation-induced chromosome aberrations was 3.12-25 mg/kg.

Identifying lower doses of resveratrol that are effective is important because a large proportion of reported research on resveratrol uses doses that are too high to be reasonably achieved in the human clinical setting. Further, there has been a desire to use food as a source of resveratrol because food could provide an economical and readily available source of resveratrol; however, effective doses of resveratrol in food need to be identified before it becomes feasible to use food therapeutically. For these reasons, a muscadine grape extract containing *trans*-resveratrol was used to determine the efficacy of using a food source for radioprotection. A muscadine grape extract containing a known quantity of *trans*-resveratrol (combined *trans*-resveratrol and *trans*-piceid) was used to develop a dose response curve using the following *trans*-resveratrol doses: 10.70, 7.13, 5.73, 2.10, 0.90 μg/kg. The previously discussed control groups were used except a muscadine grape extract only group was developed using a 5.73 μg/kg. Muscadine grape extract was administered every 24 hours for 3 days with the third dose given 30 minutes before radiation exposure. Bone marrow was collected at a time equivalent to day 1 post-irradiation and processed for cytogenetic scoring. The mean total chromosome aberration frequency ± standard deviation was determined. Overall Kruskal Wallis ANOVA was $p<0.0001$. All the dose groups showed a significant reduction in radiation-induced mean total chromosome aberration frequencies compared to the radiation only group with the

following results: 10.70 (1.19 ± 1.69), 7.13 (0.76 ± 1.24), 5.73 (0.52 ± 0.92), 2.10 (0.52 ± 0.82), and 0.90 (1.08 ± 1.49) μg/kg. These results and the statistical comparisons indicate that the optimal dose of resveratrol found in the muscadine grape extract was 2.10-7.13 μg/kg.

As a direct comparison to the muscadine grape extract (5.73 μg/kg) group, 1 mouse group was administered 5.75 μg/kg of resveratrol as a single agent before radiation exposure. The resveratrol as a single agent followed by radiation resulted in a mean total chromosome aberration frequency of 1.47 ± 1.8 which is significantly ($p<0.0001$) different than the muscadine grape extract (0.52 ± 0.92) with the same *trans*-resveratrol content. This demonstrates that when the muscadine grape extract is dosed, based on its *trans*-resveratrol content, and administered before radiation exposure, there is a significant improvement in the efficacy of reducing the radiation-induced chromosome aberrations compared to resveratrol as a single agent at a comparable dose. Further, these results indicate that the muscadine grape extract when administered based on the *trans*-resveratrol content is significantly better than any of the doses of resveratrol as a single agent used in the preceding series of experiments. These results show that the muscadine grape extract can provide sufficient quantities in reasonably achievable doses to have significant molecular effects.

The resveratrol mechanisms that contribute to the reduction in chromosome aberrations and PU.1 loss were not investigated in this study. However, there are a number of reported features of resveratrol that can provide a basis for speculation. First, resveratrol has been shown to have effects that are concentration dependent. Using 7 different human leukemia cell lines (K562, KCL22, HL60, THP1, U937, Jurkat, and

WSU-CLL), Ferry-Dumazet *et al* showed that proliferation was inhibited by different concentrations of resveratrol with the IC_{50} ranging from 5-42 μM (2). These findings were confirmed in another study using human (HL60 and U937) and mouse (32Dp210 and L1210) leukemia cell lines where the IC_{50} as found to be 18-34 μM (3). These concentration dependent effects were clearly shown in human leukemia (U937) cells exposed to resveratrol concentrations of 30-120 μM for 24 hours. There was a variable rate of cells in S phase depending on the resveratrol concentration with 30 and 60 μM resulting in 71% and 78% in S phase, respectively. Cells exposed to 90 and 120 μM did not accumulate in S phase (4). Second, as these studies demonstrate, the effects of resveratrol are cell line dependent. Each cell line exhibited a different IC_{50}. In addition, when normal bone marrow cells were treated with resveratrol it was found that they were less sensitive to the antiproliferative effects of resveratrol with an IC_{50} of 59μM (3). Third, the effects of resveratrol are time dependent. For example, human leukemia (U937) cells were exposed to 50 μM resveratrol and observed at 0, 6, 12, 18, and 24 hours. These cells showed a progressive increase in S phase accumulation through the observation period (4). With increasing exposure time (up to 3 days), human leukemia (K562 and KCL22) cells were shown to be progressively inhibited (2). Fourth, the effects of resveratrol can be reversible. When resveratrol (80 μM) was withdrawn after 20 hours of exposure, cell proliferation resumed in human leukemia (HL60) cells, mouse leukemia (32Dp210 and L1210) cells, and normal mouse bone marrow cells. There was a slight reversal (5-15%) in the leukemia cells and a substantial reversal in the bone marrow cells (60-65%) (3). This reversibility was also shown in human leukemia (U937) cell line when investigating the S phase accumulation following 24 hours of 50 μM

resveratrol exposure. During the 12 hour post-resveratrol period, a synchronous progression to G2/M phase was observed over 6-9 hours and asynchronous cell cycle patterns were observed after 12 hours (4).

Each of these points is relevant when considering the *in-vivo* investigation of resveratrol, especially when considering a tissue like bone marrow that is populated by a range of different cell types in different stages of maturation. First, the concentration of resveratrol achieved in the bone marrow and the length of time needed to develop a steady state concentration following daily administration are unknown in the mouse. Resveratrol has been shown to be present as the glucuronide and sulfate conjugates in mice at a fairly constant level (1.5 μM) for the entire 6 hour study period following a 5 mg/kg dose (5). This observation of low blood levels was confirmed in mice using a 20 and 60 mg/kg resveratrol doses over a 4 and 3 hour period respectively (6). Using a resveratrol dose of 4000 mg/kg, Horn *et al* showed that plasma concentrations of resveratrol conjugates reached a peak at 1 hour (534 ± 228 μM glucuronide and 386 ± 15.3 μM sulfate), decreased at 2 and 4 hours, and increased between 4 and 8 hours. The increase at 4 and 8 hours was attributed to enterohepatic recirculation. Resveratrol conjugates were still present at 24 hours at 25-30 μM (7). This study used an extreme resveratrol dose and it is unknown how these findings relate to a more typical dose. However, lower doses have implied that following initial uptake, resveratrol is released from the tissues back into the blood. Further, resveratrol can be added back into the circulation via enterohepatic recirculation. This point requires further investigation since mice are known to utilize corphagia. While the exact pharmacokinetics of resveratrol in the mouse bone marrow is currently undetermined, it is feasible that the bone marrow is

exposed to low levels of resveratrol conjugates over a prolonged period of time following a single dose.

The second point of interest is the difference in cell sensitivity to resveratrol. This is important because the bone marrow contains a variety of cell lines including the leukocytes, erythrocytes, and platelets maturation series. It is possible that a variable sensitivity exists among these different bone marrow cell series. Sensitivity to apoptotic agents is known to change throughout the cell cycle (8) so it is possible that sensitivity could vary in the bone marrow cells that are undergoing rapid turn over.

Based on the results from the cytogenetic and PU.1 studies reported here, it is clear that a sufficient concentration of resveratrol reaches the bone marrow and is present for a sufficient amount of time to influence molecular targets in the bone marrow cells. It is also clear that the antioxidant properties and antioxidant enzyme induction effects of resveratrol are not the paramount feature responsible for reducing radiation-induced chromosome damage because resveratrol initiation following irradiation still significantly reduces chromosome aberration frequencies and PU.1 gene loss. With this in mind, it is important to consider the effect of resveratrol on cell cycle kinetics, DNA repair, and apoptosis. Numerous studies have shown that resveratrol induces cell cycle arrest followed by apoptosis.

Cell cycle arrest has been reported to occur in G1, S, and G2/M with S phase arrest most commonly observed. This variation in cell cycle arrest phase is likely the result of the individual cell line sensitivity to resveratrol, the relative presence or absence of key proteins, the concentration of resveratrol used, and the exposure time to resveratrol (9). The difference in response to resveratrol concentrations seen in human leukemia

(U937) cells, as previously discussed, shows that lower concentrations of resveratrol (30 and 60 μM) resulted in S phase arrest while higher concentrations (90 and 120 μM) did not (4). This concentration dependent effect on cell cycle phase arrest was also illustrated in human acute promyelocytic leukemia (NB4) cells where concentrations of 6.25, 12.5, 25, and 50 μM were used for 24 hours and analyzed with flow cytometry (10). Normal human lymphoblastoid cells (AHH-1) showed a partial arrest in early S phase after 6 hours and continued accumulation in S phase with 24 hours of resveratrol (100 μM) exposure (11).

Cell cycle arrest becomes apparent in the in the first 24 hours of resveratrol exposure (2,4,12,13). The concentration and kinetics of cell cycle arrest is important when investigating the radioprotective and cancer preventive effects of resveratrol for bone marrow cells because it is known that cells vary in their sensitivity to radiation dependent on the cell cycle stage. Cells in G2/M are the most sensitive and late G1 and early S are intermediate. In human leukemia (K562, CEM-C7H2, OCIM2 OCI/AML3, U937, and HL60) cells, cell cycle arrest has occurred in the S phase but HSB-2 cells arrested in G1 while K562 cells initially accumulated in G1 then in S phase (12,14). Arrest at the G1/S transition has been shown to result from resveratrol mediated induction of p21and inhibition of cyclin D1/D2-cdk2, cyclin D1/D2-cdk4, and cyclin E-cdk2 in human epidermoid carcinoma (A431) cells (15). Consistent with the tissue specific effects of resveratrol, human prostate cancer (LNCaP) cells were shown to arrest in S phase as a result of decreased nuclear $p21^{CIP1}$ and $p27^{CIP1}$ and an increase in Cdk2 activity associated with cyclin A and cyclin E (16). In U937 cells, cyclin A and cyclin E

expression levels increased reaching a maximum at 18 hours and maintained at 24 hours after resveratrol exposure (4).

When resveratrol was administered starting 2 days before irradiation (Res+RAD), sufficient resveratrol exposure time has occurred to induce cell accumulation in the G1/S transition or early S phase. With radiation exposure and this cell synchronizing effect of resveratrol, these cells would be expected to have a higher frequency of chromosome aberrations. S phase arrest was also shown in human cervical carcinoma cell lines (HeLa and SiHa) and human non-small cell lung cancer cells (NCI-H838) as a component of a radiosensitizing effect of resveratrol (10-25 μM and 0-50 μM respectively) (17,18). This is the most likely explanation for the observation that the Res+RAD (0.093 ± 0.29) cells had the highest mean frequency of micronuclei on day 1 compared to the RAD (0.057 ± 0.23) and RAD>Res (0.065 ± 0.25) cells. The mean frequency of micronuclei is an indication of chromosome damage and apoptosis which leads to the conclusion that resveratrol sensitizes the cells to radiation-induced damage as observed at the 1 day time point. This cell cycle arrest would also limit the number of cells observed in metaphase which would be expected to result in a overall lowering of the mean total chromosome aberration frequency observed in the Res+RAD group compared to the RAD group at day 1. However, it is important to note that micronuclei can also be indicative of apoptosis (19).

Apoptosis generally follows cell cycle arrest in resveratrol exposed cells so it is important to consider how apoptosis plays a role in the observed frequency of chromosome aberrations in the Res+RAD and RAD>Res groups at the 1, 7, and 30 day end points. In studies investigating resveratrol induced apoptosis, the highest levels of

apoptosis were generally seen at 48-72 hours after resveratrol exposure and were linked to such activities as reduced expression of survivin (20, 40, and 80 μM resveratrol and primary adult T cell leukemia – ST1; 30 μM and neuroblastoma cells – SHEP), caspase 2 activation (25, 50, 75, and 100 μM and human colon adenocarcinoma cells – HT116), caspase 3 activation (12.5, 25, 50 μM and acute promyelocytic leukemia cells – NB4; 25 μM and K562 and HSB-2 cells), caspase 8 activation (25 μM and K562 cells), caspase 9 activation (50 μM and HL60 and Jurkat cells; 25 μM and K562 cells), increased Bax and Bak expression (10 μ and Burkitt's lymphoma B cells – Ramos cells; 50 μM and human colon adenocarcinoma cells – HT116), down regulation of Bcl-x(L) and Mcl-1 via inhibition of the ERK1/2 pathway (10 μ and Burkitt's lymphoma B cells – Ramos cells), and cytochrome c release (50 μM and human colon adenocarcinoma cells – HT116) in a cell line and concentration dependent manner (10,12,13,20,21,22,23).

Resveratrol has also been shown to increase apoptosis following irradiation in a dose and cell line dependent manner (24). These studies were performed *in-vitro* with chronic myeloid leukemia (K562) and multiple myeloma (IM-9) cells using resveratrol concentrations of 0-200 μM and 0-8 Gy X-irradiation. Unfortunately, cells were examined after 72 hours of resveratrol treatment and it is unclear how resveratrol influenced cell cycle distribution in these cell lines at the 24 and 48 hour end points.

When resveratrol was administered 2 days before irradiation (Res+RAD), bone marrow cells that were sensitive to induction of apoptosis would be expected to have initiated or to be undergoing apoptosis at the time of radiation exposure. These cells would be expected to have disappeared at the 1 day post-irradiation end point. However, bone marrow cells that were sensitive to radiation damage, either because of the cell type

or the cell cycle phase, would be induced to undergo apoptosis depending on the significance of the radiation-induced damage. The majority of these cells would be expected to be present at the 1 day end point and have the potential to be scored for chromosome aberrations if they had reached metaphase at the time of sampling. Apoptosis induced by resveratrol exposure would be expected to maximally occur after the 1 day end point meaning that these damaged cells would be eliminated after the 1 day time point but before the 30 day time point. The net result is that the frequency of chromosome aberrations would be higher at the 1 day end point in the Res+RAD groups compared to the RAD>Res groups but not at the 30 day end point.

When resveratrol was administered 2 hours after radiation exposure (RAD>Res 2 hrs and RAD>Res 2 hrs+), the bone marrow cells would not be in a resveratrol induced synchronized state (keeping in mind that this would only be cells sensitive to resveratrol at the concentrations achieved in the bone marrow) and would not be any more sensitive to radiation-induced injury than the RAD group. At the 2 hour post-irradiation time point, a significant amount of DNA repair has already occurred. Since the metaphase bone marrow cells observed at 1 day post-irradiation in the RAD>Res 2 hrs group had a significantly lower frequency of chromosome aberrations than the RAD group it can be assumed that resveratrol exposure altered either the cell cycle progression, the DNA repair processes, or the level of apoptosis. Since it appears that resveratrol induced cell cycle arrest is most apparent at 24 hours, it is possible that cell cycle arrest could contribute to fewer cells in metaphase at the 1 day end point. It is also possible that resveratrol has facilitated increased DNA repair. Double strand break repair surveillance genes (*BRCA1, BRCA2, and TP53*) mRNA have been found to increase with resveratrol

exposure in HBL100, MDA-MB-231, MCF-7, and MCF10A human breast carcinoma cells. In addition, resveratrol increases Rad51 protein, activates the ATM/ATR-Chk1/Chk2-Cdc25 pathway, and activates SIRT1 (25).

Resveratrol induced apoptosis would not be expected to be prominent at the 1 day post-irradiation end point. Preliminary data indicates that apoptosis at 24 hours post-irradiation is not significant in the Res+RAD and RAD>Res 2 hrs compared to the RAD group which had appreciable levels of apoptosis. If cell cycle arrest is the dominant activity of resveratrol at the 1 day end point, apoptosis would be expected to be prominent at the 48-72 hour time. This means that the unrepairable cells would be eliminated and not present at the 7 or 30 day end points. This sequence of cell cycle arrest followed by apoptosis provides a plausible explanation for the reduction in radiation-induced chromosome aberrations seen at the 1, 7, and 30 day end points regardless of when resveratrol administration was initiated.

Since the muscadine grape extract administered at a rate consistent with a *trans*-resveratrol dose in the low μg/kg range is more effective than resveratrol alone in an equivalent μg/kg dose or in the much higher mg/kg doses it is important to put these findings in perspective. Grapes have over 1600 major constituents including resveratrol (26). A number of these constituents, including piceatannol and pterostilbene, have been shown to be biologically active and to have similar molecular effects as resveratrol (27-33). These constituents may act in a synergistic manner inducing molecular effects even at the low concentrations present in this muscadine grape extract. This concept is consistent with a number of commonly used medical approaches that use multiple agents to obtain the best results. For example, multiple chemotherapy agents are used in

combination and the multimodal pain management approach has yielded improvements in patient care. A recent cancer prevention study highlighted the effects of combining a polyamine inhibitor (difluoromethlyornithine) with a nonsteroidal anti-inflammatory drug (sulindac) in colorectal cancer patients. This study showed an overall 70% reduction in adenoma recurrence when using these 2 drugs together at low doses that are ineffective alone (34). Support for the concept of synergistic actions is shown by these studies and imply that similar results could be seen with combinations of the low concentration constituents found in food.

It is also important to consider why the highest doses of resveratrol either alone or in the muscadine grape extract were not the most effective at reducing the chromosome aberrations. This is likely due to the individual bone marrow lineage sensitivity and the tissue concentration of resveratrol (or other muscadine grape extract constituents) achieved. Like resveratrol, the molecular effects of piceatannol, and pterostilbene have been shown to be concentration dependent and cell line dependent (18,25,31,33). While the 100 mg/kg resveratrol dose (Res+RAD) has the potential for a partial cell cycle arrest in early S phase, the lower doses (3.12-25 mg/kg) may not cause a cell cycle arrest or may arrest in a different phase that is more radioresistant. A lack of cell cycle arrest in a radiosensitive phase or no arrest would result in less radiation-induced chromosome damage. As a result, the predominant molecular effect would be DNA repair and apoptosis, depending on the degree of DNA damage and cell sensitivity to resveratrol. This would again reduce the number of damaged cells progressing into mitosis. Since the resveratrol dose is low, resveratrol may not be influencing DNA repair or apoptosis in the same fashion as the higher resveratrol doses.

Another consideration is that both the resveratrol and muscadine grape extract dose response curves show a biphasic patterns with both the highest and lowest doses of resveratrol having the least effect on reducing chromosome aberration frequencies. Hormesis is a concept that describes the observation that low doses of substances result in stimulation while high doses result in inhibition in a dose-time relationship (35,36). This concept refers to a frequent observation that at low doses there can be an increase in multiple parameters including cell numbers or viability relative to controls. The maximum stimulatory response is generally 30-60% greater than the control and is thought to represent a cellular attempt to re-establish homeostasis. As would be expected, the hormesis concept should not be applied to nutrients like vitamins and minerals because the cellular response could represent responses to nutrient excesses or deficiencies. However, resveratrol is not considered to be a nutrient. With the time points used in the study reported here and the variables inherent with the multiple bone marrow cells involved, the radiation effects, and the continuum of concentration related effects of resveratrol, it is difficult to determine the true impact of the hormesis phenomenon on these results. In order to define how hormesis relates to the reductions in radiation-induced chromosome aberrations, studies specifically designed to investigate this would have to be designed and would require a more complete assessment of the bone marrow and bone marrow cell lineages as they relate to kinetic changes induced by radiation exposure, resveratrol exposure, and combined radiation and resveratrol exposure. Without these additional studies, the results reported here can be explained through the concentration and cell line dependent features associated with resveratrol that have already been discussed.

The studies reported here provide a foundation for future investigation of the mechanisms that underlie the observation that resveratrol and the muscadine grape extract have radioprotective effects and have the potential to reduce the incidence of radiation-induced cancer. Establishing a clear understanding of the mechanisms requires an examination of bone marrow cell cycle kinetics, apoptosis kinetics, pharmacokinetics of resveratrol in bone marrow, and the incidence of AML in irradiated CBA mice.

Pharmacokinetic studies will define the time and dose dependent features of resveratrol concentrations in the bone marrow cavity. This information will provide a link to *in-vitro* resveratrol studies and guidance for selection of *in-vivo* dosages. Resveratrol doses of 100 mg/kg and 3.12 mg/kg should be used with tissue sampling at 30 min, 24 hours, 72 hours, and 30 days should be included. Tissues of interest are blood, liver, kidney, spleen, and bone marrow. Urine should be collected 0-1, 2-3, and 5-6 hours after resveratrol administration. The sample tissue samples and time should be used for the muscadine grape extract that delivers 5.73 μg/kg. Compounds of interest in the pharmacokinetic studies include resveratrol, resveratrol sulfate, resveratrol glucuronide, piceatannol, piceatannol sulfate, piceatannol glucuronide, pterostilbene, pterostilbene sulfate, and pterostilbene glucuronide.

Bone marrow cell cycle studies should be used to define how these resveratrol (100 mg/kg and 3.12 mg/kg) and muscadine grape extract (5.73 μg/kg) doses affect cell cycle kinetics of the bone marrow in general and specific bone marrow cell lineages. These cell cycle studies should examine 6, 12, 24, and 48 hour time points after resveratrol administration. Cell sorting should be employed to focus on the granulocyte cell line. In addition, if cell accumulation occurs in a specific cell cycle phase,

measurement of proteins of importance in that phase should be examined. For example, Western blot studies can be performed on cyclin D1 or phosphorylated retinoblastoma protein for cells that arrest in the G1/S transition.

The kinetics of apoptosis in bone marrow as a whole, and with specific bone marrow cell lineages should be investigated with time points of 12, 24, 48, and 72 hours after resveratrol administration. These studies could be performed using flow cytometry and 100 mg/kg and 3.12 mg/kg doses of resveratrol and 5.73 μg/kg resveratrol in the muscadine grape extract. Proteins of interest include activated caspase-3, Bax, and Bcl-2.

The use of muscadine grape extract administered at μg/kg doses of *trans*-resveratrol, clearly demonstrated the ability of the muscadine grape extract to reduce radiation-induced chromosome aberrations in bone marrow cells. Since the equivalent dose of resveratrol as a single agent was significantly inferior at reducing these chromosome aberrations, additional studies investigating individual muscadine grape extract constituents should be performed. Understanding the cell cycle and apoptosis kinetics following muscadine grape extract administration would facilitate recognition of the temporal relationships between cell cycle arrest and apoptosis. Studies of the muscadine grape extract should include investigation of piceatannol and pterostilbene as single agents in the same way that resveratrol was investigated here.

Incidence studies of AML in CBA mice when resveratrol is initiated before or after irradiation should be undertaken to determine if the radiation-induced cancer prevention that is implied here is supported. Studies using both resveratrol (100 mg/kg and 3.12 mg/kg) and the muscadine grape extract (5.73 μg/kg *trans*-resveratrol) should be used. In addition, since a single dose of resveratrol 2 hours after irradiation

(RAD>Res 2 hrs) showed a reduction in chromosome aberrations at 1 and 30 days, incidence studies using a single dose should be included. The results of these studies would define the optimal timing, in relationship to the timing of radiation exposure, of resveratrol administration for reducing the incidence of AML in these mice. In addition, because irradiated CBA mice are prone to developing hepatic carcinoma, the incidence of this cancer could be monitored.

Demonstrating an influence by resveratrol or muscadine grape extract on the incidence of AML, would facilitate correlation of the findings in the cytogenetic, PU.1 gene loss, and proposed cell cycle and apoptosis studies. This is a crucial step in developing a clinical application for resveratrol. An example of a clinical application would be the use of resveratrol in radiation therapy patients following their course of therapy with the goal of reducing the incidence of therapy related cancer such as AML. This is important because therapy induced AML can be difficult to treat. Since these human studies would require a large number of patients and a long time course, it is important to establish appropriate intermediate biomarkers so that periodic assessment of the patients can occur.

It is clear that resveratrol administration before or after radiation exposure results in a reduction in chromosome aberration frequencies and PU.1 gene loss. These observations are the result of a sequential pattern of cell behavior induced by resveratrol exposure. Initial cell cycle arrest is followed by DNA repair or apoptosis depending on the significance of the DNA damage. The cell cycle arrest likely progresses from a partial G1/S transition arrest to an accumulation of sensitive cells in S phase. Arrest in S phase has the potential to improve the quantity and quality of DNA repair before the cell

enters mitosis. Cells that cannot be effectively repaired would undergo apoptosis. Resveratrol can induce signaling pathways that induce expression of proteins that facilitate cell cycle arrest and apoptosis. Therefore, it is likely that resveratrol activates MAPK pathways that lead to increases in p53 via p38 or ERK which can lead to both cell cycle arrest and apoptosis. p53 activation can lead to induction of p21 which has an inhibitory effect on cyclin D1 and cyclin E and p53 can also play a role in induction of apoptosis through proapoptotic proteins like Bax. Regardless of the success of DNA repair, the cell cycle arrest or apoptosis would prevent damaged cells from undergoing duplication and would reduce the potential pool of cells that could undergo proliferation and lead to AML. As a result of the ability of resveratrol to induce a cascade of molecular events that promote DNA repair and cell survival if the DNA damage is sufficiently repairable or to induce apoptosis if the DNA damage is not repairable give resveratrol the potential to be an important part of patient management for prevention of therapy related AML.

This study fulfilled its aims by demonstrating that resveratrol could reduce the frequency of radiation-induced chromosome damage in mouse bone marrow cells and the loss of the PU.1 gene. Resveratrol provides this effect when given before or after irradiation. Optimal doses of resveratrol for administration before radiation exposure were shown to be in a reasonably achievable range. In addition, the muscadine grape extract was shown to be significantly more efficient at reducing the frequency of radiation-induced chromosome aberrations when administered before irradiation. The muscadine grape extract was also shown to reduce the loss of the PU.1 gene. Taken together these results indicate that the muscadine grape extract can provide protective

effects in doses that can be achieved. Future studies will continue to define the mechanisms that are responsible for these important observations with the goal of translation into clinical applications.

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