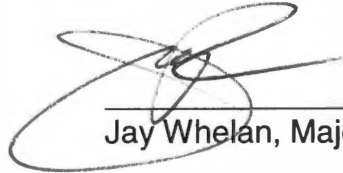


To the Graduate Council:

I am submitting herewith a thesis written by Carol Ziegler entitled "Effects of resveratrol on tumor load, tumor COX-2 expression and tumor PGE₂ biosynthesis in a murine model of intestinal tumorigenesis." I have examined the final paper copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Masters of Science, with a major in Nutrition.

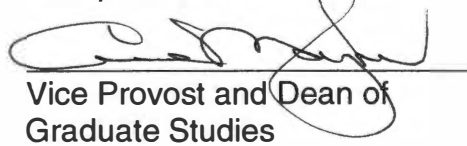


Jay Whelan, Major Professor

We have read this thesis and
recommend its acceptance:



Acceptance for the Council:



Vice Provost and Dean of
Graduate Studies

**Effects of resveratrol on tumor load, tumor COX-2 expression and tumor PGE₂
biosynthesis in a murine model of intestinal tumorigenesis**

A Thesis
Presented for the
Masters of Science
Degree
The University of Tennessee, Knoxville

Carol Cathleen Ziegler
December 2002

DEDICATION

Thesis
2002
.Z7

I dedicate this thesis to mom, dad, Laura and Rhonda.

ACKNOWLEDGEMENTS

I sincerely thank all those who contributed to this project and helped me in the completion of a Master of Science in Nutrition. I thank everyone who served on this committee. First, I thank Dr. Whelan for my education, for all of his guidance, time and resources invested into my research, for allowing me the privilege of working in his laboratory, and for his invaluable contributions to the design of the research project and manuscript. I also thank him for being an ideal mentor professionally and personally. I thank Dr. McEntee for securing the funding for this project and allowing me the opportunity to work on it, for his invaluable contributions to the design of these experiments and the completion of the manuscript, and for teaching me how to conduct immunohistochemical staining of tumors. I thank Dr. Moustaid-Moussa for her ideas and contributions to the development of the COX-2 activity assay, and for her encouragement and valuable input in my research presentations along the way. I thank Leah Rainwater for conducting immunohistochemical staining of the tumors.

I also wish to thank Dr. Melissa Hansen-Petrik and Ben Johnson for their time, effort and encouragement in teaching me various laboratory protocols.

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CHAPTER I

REVIEW OF THE LITERATURE

Introduction

Colorectal cancer is one of the leading causes of cancer-related death in the United States. Although a small percentage of human colorectal cancers result from inherited gene defects which confer a strong predisposition for tumor development, such as in familial adenomatous polyposis (FAP) and hereditary nonpolyposis colorectal cancer (HNPCC), the majority of cases occur sporadically as a result of the accumulation of somatic mutations. Mutations in the *adenomatous polyposis coli* (*APC*) tumor-suppressor gene are responsible for FAP and are observed in a majority of sporadic colorectal cancers, making animal models with *Apc* gene defects ideal for studying this disease process. Several animal models have been developed with targeted *APC* mutations, and these models are useful in determining the effects of potential anti-tumorigenic treatments in *APC*-driven intestinal tumorigenesis. The *Apc*^{Min/+} mouse model is a murine model of human FAP in which intestinal tumors develop spontaneously throughout the intestinal tract that are sensitive to modulation of the arachidonic acid pathway via cyclooxygenase (COX) inhibition. COX catalyzes the committed step in the conversion of arachidonic acid to prostaglandins. There are two isoforms of this enzyme, known as COX-1 and COX-2. COX-1 is constitutively expressed in most tissues and is believed to be a housekeeping enzyme involved in maintaining homeostasis. COX-2 is inducible in inflammatory conditions and catalyzes the formation of prostaglandins involved in the

promotion of tumorigenesis. Prostaglandins can be pro-inflammatory and appear to be important in tumor progression. Intestinal tumors over-express COX-2, and it has been proposed that this up-regulation confers a growth advantage to tumors by promoting angiogenesis.

Resveratrol, a polyphenolic phytochemical found in red wine, has been demonstrated to inhibit tumor growth in vitro in several immortalized human colorectal cancer cell lines (1) and to inhibit COX-2 in human colorectal cancer cells expressing mutated *APC* (2). However, discordant effects have been reported regarding resveratrol's efficacy as a potential anti-tumorigenic agent in vivo. While resveratrol inhibited the formation of aberrant crypt foci (ACF) in a carcinogen-induced rodent model of colorectal cancer (3), it had no effect in a murine xenograft model of leukemia (4). Therefore, our study was designed to evaluate the anti-tumorigenic effect of dietary resveratrol in *Apc^{Min/+}* mice. These mice (6-7 animals per group) were fed diets containing increasing levels of resveratrol (4-90 mg per Kg BW) for seven weeks. Animals fed resveratrol at 90 mg per Kg BW had significant reductions in tumor biosynthesis of PGE₂. However, resveratrol had no effect on tumor number or tumor COX-2 expression. Therefore, while resveratrol is a potent inhibitor of cell growth in vitro and inhibits PGE₂ biosynthesis in vivo, it was unable to reduce tumor load in *Apc^{Min/+}* mice.

Colorectal Cancer

Background Information

Colorectal cancer is currently the third-leading cause of cancer-related death in the United States for both males and females, and resulted in 56,700 deaths in 2001 (5). It is estimated that 135,000 new cases of colorectal cancer are diagnosed each year (5), primarily in persons sixty years of age or more. Approximately 50% of persons in Western populations will acquire a colorectal tumor by the age of 70 as a result of the accumulation of somatic mutations over time (5-6). These sporadic colorectal cancers account for 90% of all colorectal cancers, while the remaining ten percent occur earlier in life in families with germline defects resulting in strong predispositions for tumor development (7).

Sporadic Colorectal Cancers

Sporadic colorectal cancer is typically diagnosed in the sixth or seventh decade of life, resulting in a decreased tumor load relative to the hereditary colorectal cancers. Although most sporadic adenomas (90%) remain benign, ten percent will progress to malignancy within five to ten years once they acquire characteristics allowing them to penetrate the basement membrane and metastasize to other tissues via the blood or lymphatic fluid (8-10). Several factors have been identified that may increase the likelihood of developing sporadic colorectal cancer, including a history of inflammatory bowel disease, age (> 50 years), a high-fat, low-fiber diet, physical inactivity and obesity (11-15). Although various mutations have been identified as contributors to sporadic

colorectal cancer, mutations in *APC* are observed in approximately 80% of all sporadic colorectal cancers.

Hereditary Colorectal Cancers

HNPCC, the most common form of hereditary colorectal cancer, accounts for eight percent of all human colorectal cancer incidence (16). HNPCC is generally diagnosed in the third or fourth decade of life and results in the presence of multiple tumors, mostly in the proximal colon, which will most likely progress to carcinoma (17). Extra-colonic tumors such as endometrial, ovarian, and gastric tumors are also observed in these patients (17). Although mutations in several genes, including *APC* have been observed in HNPCC (18-21), the majority of mutations occur in genes involved in the correction of base-base mismatch errors, known as the mismatch repair system (7-8).

When a base-base mismatch occurs, the error is recognized by the *Mut S* homolog (MSH) complex, which recruits the *Mut L* homolog (MLH), a repairing protein, to the site. Exo-nucleases remove the mismatched base, and a corrected strand is inserted into the original strand by DNA polymerase and DNA ligase. In HNPCC, loss of heterozygosity in either MSH (40% of cases) or MLH (50% of cases) results in defects in the mismatch-repair system, increasing the survival of downstream pro-tumorigenic DNA replication errors by 100 to 1000 fold and promoting tumor development (22-24).

FAP occurs in families with a strong predisposition to colorectal cancer development as a result of a germline mutation at chromosome five in one allele

of the *adenomatous polyposis coli* (*APC*) tumor suppressor gene (25-30).

Adenoma formation results when a somatic mutation occurs in the remaining wild-type allele, resulting in loss of heterozygosity and translation of a non-functional, truncated *APC* protein product (27,31). FAP accounts for only one percent of all heritable colorectal cancers but results in a 100% chance of developing hundreds to thousands of colorectal adenomas by the second decade of life with a high likelihood of progression to adenocarcinomas (32-33).

Depending on the location of the *APC* mutation, extra-colonic tumors may develop in the form of retinal lesions, osteomas, skin desmoids, and brain tumors (35-36). The location of the mutation also dictates the age of onset and the severity of the disease (36-40). It appears that mutations occurring in the center regions of *APC* (known as the mutation cluster region) result in the most severe phenotypes, while mutations in the first third and last third of the gene are associated with decreased severity (41-42).

FAP may be categorized into three phenotypes (based on severity) as profuse, severe and attenuated FAP (**Table I-1**, p.6). Profuse FAP is the most severe phenotype, and results in the formation of greater than 5,000 colonic tumors due to a mutation at exon 15, between codons 1255 and 1467 in the center of *APC*, (42). In severe FAP, the *APC* mutation occurs between codons 600-1600 and result in the formation of 2,000-5,000 colonic tumors (43). In the least severe form, attenuated *APC* (AAPC), mutations occur upstream of codon 200 or downstream of codon 1600 of *APC*, resulting in the formation of 100-2,000 polyps (41, 44-48). The decreased severity observed in AAPC is due

Table I-1. FAP Phenotypes and Characteristics.

Syndrome	Mutation Location (codon)	# Tumors
Profuse FAP	1255-1467	> 5,000
Severe FAP	600-1600	2,000-5,000
Attenuated FAP	200-1600	100-2,000

to the location of the mutatin towards the outer ends of *APC*, allowing the expression of a functional protein product (49).

Adenomatous Polyposis Coli

APC is a tumor suppressor gene expressed by non-dividing cells at the upper region of the crypts and along the villus of the small intestine and colorectal epithelium (50). It functions as a regulator of intestinal cell migration and cell-cell adhesion, and when *APC* is truncated in normal intestinal tissue, in vivo cell migration is inhibited, resulting in tumor formation (51-53).

The *APC* mutation is believed to be an early event in intestinal tumorigenesis, and mutations in this gene are frequently observed in tumors from sporadic, HNPCC, and FAP patients (29, 35, 54-60). More than 80% of colonic tumors taken from sporadic colorectal cancer patients and 100% of tumors taken from FAP patients express mutated *APC* (61). The relatively high frequency of *APC* mutations observed in various forms of colorectal cancers and the early role

of *APC* in the tumorigenic process suggest that animal models with germline *Apc* mutations would be ideal in studying *Apc*-driven intestinal tumorigenesis.

APC regulates cell-cell adhesion and cell migration by promoting the degradation of the transcriptional activator, β -catenin, through the *Wnt* signaling pathway (62-64). Nuclear β -catenin is an activator of pro-tumorigenic transcription factors and its expression is increased in primary and metastatic tumors from sporadic colorectal cancer patients (66). Mutations in *APC* frequently occur at the β -catenin binding domains, altering the ability of *APC* to regulate β -catenin via the *Wnt* signaling pathway (**Figure I-1**, p. 8) (66). In this pathway *Wnt* binds to its cell surface receptor, frizzled (*fzd*) (67) and activates disheveled (*dsh*), which then inactivates glycogen synthase kinase-3- β ($GSK_3\beta$) (63). Active $GSK_3\beta$ forms a complex with *APC* and its scaffolding protein, axin, and this complex facilitates $GSK_3\beta$ -mediated phosphorylation of β -catenin, earmarking it for ubiquitination by the E_3 ubiquitin ligase and subsequent degradation in the proteosomes (68). When $GSK_3\beta$ is inactivated by the *Wnt* signaling cascade, β -catenin accumulates in the cytosol and eventually translocates to the nucleus where it promotes the activation of pro-tumorigenic transcription factors such as T-cell factor/lymphoid enhancer factor (*Tcf/Lef*) (63); (68-72). Animal models of FAP, such as the *Apc*^{Min/+} mouse, have increased expression of β -catenin due to the defect in *Apc*. Non-steroidal anti-inflammatory drugs (NSAIDs), proven to be effective in inhibition of tumorigenesis in these animals, normalize expression of β -catenin (73).

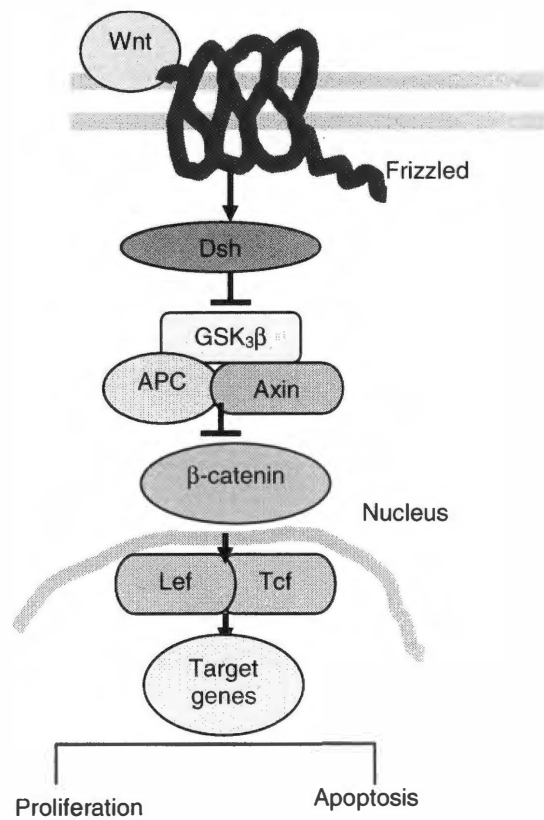


Figure I-1. The *Wnt* Signaling Cascade

Models of Intestinal and Colorectal Cancer

Several animal models are used in the study of the initiation, progression and treatment of colorectal cancer. Popular models of colorectal cancer and their characteristics may be found in **Table I-2** (p.10).

C57BL/6J *APC*^{Min/+} Mouse

A murine model of human FAP was derived through an *N*-ethyl-*N*-nitrosurea (ENU)-induced mutation at the 15th exon of murine *Apc* in a C57BL/6J mouse. This mutation resulted in the formation of multiple intestinal neoplasia (min), and thus the *Apc*^{Min/+} mouse model (31, 74). The *Apc*^{Min/+} mouse carries a nonsense mutation in *Apc* at nucleotide 2549, substituting TTG with a stop codon (TAG) (75). This substitution results in the truncation of the *Apc* protein from 300kD to 95kD, and subsequent protein dysfunction leading to tumor development (31, 45). These mice develop 40-50 adenomas, mostly in the small intestine, which typically do not progress to carcinomas. However, this severe tumor load results in a shortened lifespan of up to 150 days due to anemia or intestinal obstruction (75). The *Apc* defect in the *Apc*^{Min/+} mouse makes it a useful model in studying colorectal cancer, since this defect is present in 100% of human FAP and 80% of human sporadic colorectal cancer patients (56, 76).

Other models of *Apc*-directed tumorigenesis

Other models of *Apc*-directed tumorigenesis have been developed through target mutations in different regions of *Apc*, resulting in some variations in phenotype, but these animals typically display similar patterns of

Table I-2. Animal Models of Human Colorectal Cancers and their Characteristics

Animal Model	Earliest Genetic Defect	Tumor Location	Tumor Multiplicity	Aberrant Crypt Foci	Neoplastic Progression
AOM	<i>β-catenin, Ras>>>A</i>	Colon	< 1-3 per animal	Yes	Yes
<i>Apc</i>^{Δ1638T}	<i>Apc</i>	None	None	No	N/A
<i>Apc</i>^{Δ1638N}	<i>Apc</i>	Small Intestine, Follicle, Skin	5-6	No	No
<i>Apc</i>^{Δ474}	<i>Apc</i>	Intestine & Breast	≥ 30 per mouse	No	No
<i>Apc</i>^{Δ580}	<i>Apc</i>	None	None	No	N/A
<i>Apc</i>^{Δ716}	<i>Apc</i>	Small Intestine > Colon	200-300	No	No
<i>APC</i>^{Min/+}	<i>Apc</i>	Small Intestine > Colon	≥ 30 per mouse (< 4 colonic)	No	No

tumorigenesis as those observed in the *Apc*^{Min/+} mouse. A mutation at amino acid 716 of *Apc* results in the formation of the *Apc*^{Δ716} mouse model (77).

Homozygotes are embryonic lethal, and by three weeks of age, heterozygotes develop micro-adenomas from the duodenum to the rectum. By sixteen weeks of age, 200 to 300 polyps are present, predominantly in the small intestine, ranging in size from 0.2 to 5 mm, and these polyps are sensitive to selective COX-2 inhibition and PGE₂ receptor (EP₂) antagonism (79-80).

Mutations in *Apc* at exon 10 result in the truncation of the protein at 474 amino acids and the formation of the *Apc*^{Δ474} mouse model. These animals have a phenotype similar to the *Apc*^{Min/+} mouse with the exception of the presence of mammary tumors in 18% of the animals and tumors in these animals are also sensitive to COX-2 inhibition (81).

Apc^{Δ1638} mice were developed through a targeted mutation at amino acid 1638 on *Apc* (82). Two forms of this model exist; *Apc*^{Δ1638N} and *Apc*^{Δ1638T}.

Apc^{Δ1638N} animals have no detectable Apc protein expression, resulting in fully penetrant multifocal cutaneous follicular cysts and desmoids in addition to attenuated polyposis of the upper GI tract (82). *Apc*^{Δ1638T} animals express a truncated protein product of 182 kD, but both heterozygotes and homozygotes are tumor-free, with no increased predisposition for tumor development, suggesting that truncation of APC at 182 kD results in production of a functional protein (83).

The Azoxymethane-induced Model of Colorectal Cancer

The azoxymethane (AOM)-induced model of colorectal cancer is derived through the administration of a bolus dose of the colon carcinogen AOM, resulting in a sequence of somatic mutations which lead to the formation of precancerous lesions, or aberrant crypt foci (ACF) in the colon. If left untreated, these ACF will progress to colorectal adenocarcinomas (82-84). AOM models typically develop one to three tumors after four weeks of treatment with 10 mg AOM per Kg body weight. Mutations in *Apc* and *β-catenin* are frequently present in tumors in this model (85), and like the *Apc*^{Min/+} mouse, AOM-induced tumors also over-express COX-2 and are sensitive to COX inhibition and PGE₂ receptor antagonism (86).

The Arachidonic Acid Cascade and Intestinal Tumorigenesis

Introduction

The enzymatic action of cyclooxygenase (COX) on arachidonic acid results in the formation of prostaglandins, which have been implicated in the promotion of intestinal tumorigenesis (87). Arachidonic acid (20:4 n-6), whether obtained directly from the diet or synthesized from linoleic acid (18:2 n-6) is stored in its esterified form in cell membrane phospholipids. Arachidonic acid is cleaved from membrane phospholipids by various phospholipases, namely cytosolic phospholipase A₂ (cPLA₂), and serves as the substrate for COX in the formation of eicosanoids (88). The rate-limiting step in the formation of the pro-tumorigenic prostaglandins is the release of arachidonic acid from membrane

phospholipids by various phospholipase A₂s. Initially, cytosolic phospholipase A₂ (cPLA₂) binds to the membrane phospholipid where it cleaves the sn2 ester bond linking arachidonic acid to the glycerol backbone. cPLA₂ has been implicated in the promotion of colorectal tumorigenesis through its role in providing the substrate for the formation of pro-inflammatory prostaglandins (89). When cPLA₂ is knocked out in *Apc*^{Δ716} mice, intestinal polyp size is decreased, but no effects on intestinal tumor number are observed (90). However, in *Apc*^{Min/+} mice, the absence of cPLA₂ expression results in an 83% decrease in small intestinal tumor number compared to animals expressing normal cPLA₂ (91). These effects are similar to decreases observed in dual *Apc*^{Min/+}/*COX-2*^{-/-} mice.

Cyclooxygenase

COX catalyzes the irreversible step in the formation of prostaglandins from arachidonic acid in a two-part cyclooxygenase-peroxidase reaction (**Figure I-2**, p.14). In the first reaction, arachidonic acid binds to the cyclooxygenase active site. A tyrosyl radical (Tyr-385) abstracts the pro-S hydrogen at C13, generating an endoperoxide via radical rearrangement. Following further rearrangement to C15, the radical attacks molecular oxygen, generating PGG₂, a peroxide intermediate. PGG₂ migrates to the peroxidase active site where it is reduced to PGH₂, the parent compound for further conversion to thromboxane, prostacyclin, or prostaglandins by the action of specific synthases.

Two isoforms of COX exist, which share 60% homology between and within species, but differ in their patterns of expression (92). COX-1 is

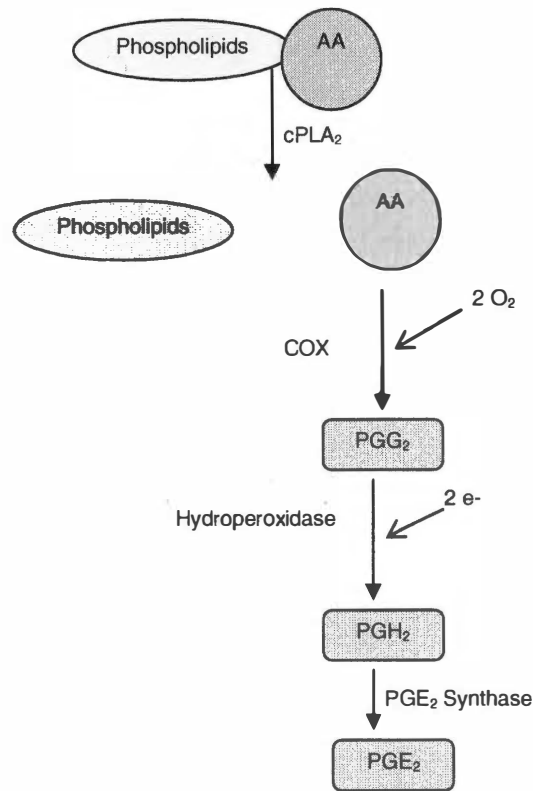


Figure I-2. The Arachidonic Acid Cascade

constitutively expressed in most tissues as a housekeeping enzyme and is involved in maintaining tissue integrity under normal conditions. This function is evident by the increased incidence of gastric ulceration following COX-1 inhibition (93-95). The physiological role of COX-1 in intestinal tumorigenesis was investigated through animal knockout models. When COX-1 was knocked out in C57BL/6J mice, animals had a normal lifespan, normal fertility, decreased platelet aggregation and were less sensitive to radiation injury compared to controls, but their offspring were embryonic lethal (96). When COX-1 was knocked out in *Apc^{Min/+}* mice, tumor load was decreased by 80%, and PGE₂

biosynthesis was decreased by 99% (97-98). However, no signs of gastric ulceration or kidney anomalies were observed.

COX-2 is constitutively expressed in the brain and kidneys, but is inducible in other tissues by pro-inflammatory cytokines, growth factors, and tumor promoters (92). Induced expression of COX-2 has been reported in human prostate and gastric tumors as well as in intestinal tumors (99) from hereditary and sporadic human colorectal cancers (100-103), and this phenomenon is observed in 85% of adenocarcinomas and in 50% of benign polyps. COX-2 expression is also induced in the intestinal tumors of *Apc^{Min/+}* mice (104) and in the carcinogen-induced colonic tumors of rodent models (105).

Selective targeting of the COX-2 gene results in survival of only 60% of pups afflicted with kidney anomalies leading to end-stage-renal disease and female infertility (98). When COX-2 is knocked out in *Apc^{Min/+}* mice, tumors are decreased by 75% compared to controls (106) and tumorigenesis in dual *Apc^{Δ716}/COX-2* null mice is reduced by 34% compared to controls (107). Inhibition of COX-2 has proven to be effective in vitro and in vivo in the prevention of colorectal cancer (108-110). NSAID-induced selective COX-2 inhibition in *Apc^{Min/+}* mice results in an 80%-86% decrease in polyps as compared to controls (111). Increased expression of COX-2 is associated with increased angiogenesis and cell motility through a matrix, two factors which are critical for the growth and metastasis of tumors (112). Selective COX-2 inhibition with NSAIDs attenuates the increased risk of angiogenesis and metastasis associated with induction of COX-2 in human colorectal cancers (113), and

induces apoptosis of human colorectal cancer cells in vitro (112, 114-115). The promotion of angiogenesis and tumor integrity by COX-2 induction in tumors is due in part to the production of prostaglandins, which promote the expression of pro-angiogenic factors such as vascular endothelial growth factor (VEGF) (111). Selective inhibition of COX-2 expression in a murine xenograft model of Lewis lung carcinoma cells resulted in a significant reduction in tumor growth and a 94% reduction in expression of VEGF (116).

Decreases in tumorigenesis achieved with the administration of NSAIDs in animal models of human colorectal cancer are related to the inhibition of COX and subsequent decreases in the biosynthesis of prostaglandin E₂ (PGE₂). NSAIDs inhibit the formation of prostaglandins by inhibiting COX (117), decreasing tumor load in *Apc*^{Min/+} mice (118). In animal models of human FAP, COX inhibition with NSAIDs decreases *APC*-mediated intestinal tumorigenesis by 85-99% (119-121). Similar effects are observed when COX is inhibited in carcinogen-induced models of colorectal cancer (122-123), and decreases in PGE₂ synthesis have been correlated with increased responsiveness to chemotherapy in human FAP patients (124). The role of PGE₂ in the promotion of tumor integrity was bolstered by the rescue of *Apc*^{Min/+} mouse tumors from NSAID-induced regression with administration of PGE₂ (125), and the acceleration of intestinal polyposis via PGE₂ receptor agonism in *Apc*^{Δ716} mice (126). However, NSAID-induced regression of ACF and *APC*-driven tumors is not totally dependent on PGE₂ synthesis (120, 127-129). Reductions in intestinal tumors of *Apc*^{Min/+} mice by 95% with sulindac, were not associated with any

significant changes in PGE₂ in normal tissue (121), suggesting that tumorigenesis is not entirely dependent on prostaglandin levels and that there is a prostaglandin-independent mechanism in driving NSAID-induced tumor inhibition.

Phytochemicals

Epidemiological evidence indicates that increased consumption of fruits and vegetables may decrease the risk for developing colorectal cancer (130-131). It is believed that in addition to the high fiber and low fat content of these foods, phytochemicals may be partly responsible for the beneficial health effects (132). Structures of several phytochemicals recently investigated for their chemopreventive properties are illustrated in **Figure I-3**, (p. 18).

In the human diet, the majority of these beneficial phytochemicals are found in fruits and vegetables, wine and tea, and soy products, with average daily intakes ranging from one to two grams (133). Extracts from black tea and red wine have been shown to decrease intestinal carcinogenesis in an AOM-induced rat model of colorectal cancer (134-135). Evidence suggests that dehydrated red wine solids contain high levels of phytochemicals which may display chemopreventive properties. Red wine solids have been shown to detoxify carcinogens in vitro and delay tumor growth in a carcinogen-induced model of colorectal cancer (136-138). These red wine solids consist of various polyphenolic phytochemicals, and estimated levels of total polyphenols in red wine range from 100 to 300 mg per liter, with the highest levels being found in Cabernet Sauvignon wines (**Table I-3**, p. 18) (139-141).

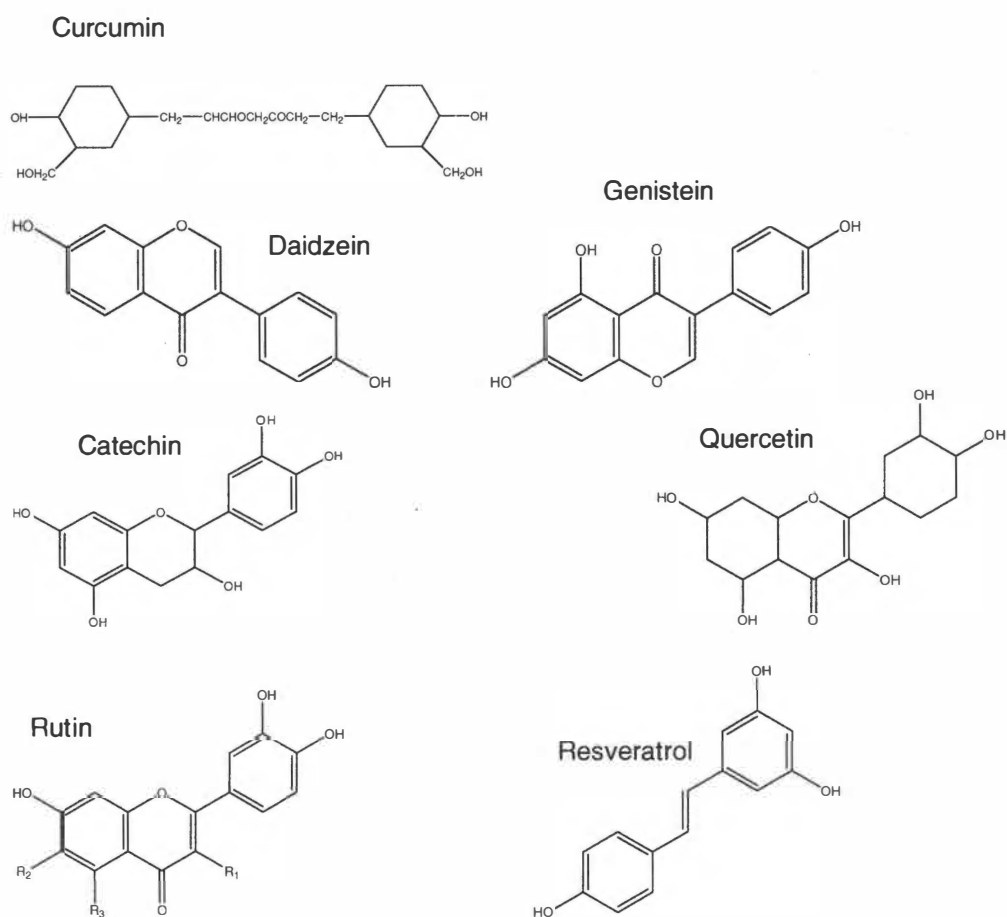


Figure I-3. Structures of Polyphenolic Phytochemicals

Table I-3. Phytochemical Profile of Red Wine

Phytochemical Classification	Quantity (mg per ml)
Cinnamates (Cinnamic Acid)	4-80
Gallates (Gallic acid)	20-40
Flavanols (Resveratrol)	40-80
Catechins	75-115
Malvidins	60-110

Numerous studies indicate that phytochemicals inhibit tumorigenesis in several immortalized cell lines and in animal models of colorectal cancer (142-144). Two soy isoflavones, genistein and daidzein, prevented the formation of aberrant crypt foci in a carcinogen-induced rodent model of colorectal cancer (145-146). Curcumin, quercetin and rutin inhibited the formation of ACF in AOM-induced rodent models of colorectal cancer (84, 147-150). Studies conducted in the *Apc^{Min/+}* mouse model indicate that catechin and curcumin decreased intestinal tumorigenesis. However, in the same study, quercetin and rutin had no effect.

Resveratrol

Resveratrol is a naturally occurring polyphenolic compound that is found in relatively high quantities in peanuts and in the skins of grapes used in preparing red wines (151-155). Levels of this compound in red wine range from 0.3 to 7.17 mg/L (156-157). Resveratrol is synthesized in plants in times of stress, offering protection from fungal infections, by the action of resveratrol synthase on coumaroyl CoA and malonyl CoA; a reaction that produces resveratrol, CoA, and CO₂ (158-159) (**Figure I-4**, p. 20).

Resveratrol and Tumorigenesis

Resveratrol inhibited the growth of several immortalized human colorectal cancer cell lines and inhibited tumorigenesis in animal models of human colorectal cancer (186-187). Several mechanisms have been proposed to explain

4 Coumaroyl CoA + 3 Malonyl CoA



Resveratrol + 4 CoA + 4 CO₂

Figure I-4. Synthesis of Resveratrol

these effects, including antioxidant and antimutagen activity, inhibition of cell cycle progression and modulation of the arachidonic acid cascade (162, 186-187).

Antioxidants inhibit carcinogenesis by protecting cells from the DNA-damaging effects of free radicals in vitro and in vivo (160-161). Resveratrol possesses potent antioxidant activity compared to α -tocopherol in Cu^{1+} induced human LDL oxidation (162) and in vitro, resveratrol prevented free-radical-induced damage to DNA and RNA (163-166). Free radical formation induced by 12-O- tetradecanoylphorbol-13-acetate (TPA) in human promyelocytic leukemia cells is inhibited by resveratrol in a dose-dependent manner (167).

Resveratrol prevents cellular damage by detoxifying carcinogens in vitro (168-171). In vivo, resveratrol prevents the development of ACF induced by 7,12-dimethylbenzanthracene (DMBA) in a dose-dependent manner (172-173). This inhibition of carcinogen-induced tumor initiation is believed to occur via a cytochrome p450 pathway. Resveratrol has been demonstrated to competitively bind to the aryl hydrocarbon receptor (AhR), antagonizing the transcription of

pro-tumorigenic genes such as CYP1A1 induced by AhR ligands, such as DMBA and TPA in vitro (174-175).

In addition to antioxidant and antimutagenic activity, many in vitro studies report that resveratrol induces apoptosis and inhibits the growth of colorectal cancer cells by disrupting cell cycle progression (**Figure I-5**, p. 22) at the S/G2 phase transition (176-182). Cyclins are synthesized continuously, accumulating as the cell cycle advances, and are destroyed at defined points. As the cyclins build up, they signal progression into the next phase of the cell cycle, acting as the 'internal timekeepers' of the cell. For entry into the S-phase, the cell must execute DNA synthesis and mitosis. Resveratrol may impede tumor cell proliferation by causing an arrest in the S-phase of the cell cycle, disrupting the S- to G2 phase transition as demonstrated by an absence of G2/M phase-associated cyclins and an increase in G1 and S phase-associated cyclins, forcing cells to undergo apoptosis (180-181, 183-189).

As mentioned previously, inhibition of COX by NSAIDs inhibits tumorigenesis in several models of human colorectal cancer, and the antitumorigenic effects attributed to resveratrol may be mediated through its ability to modulate the formation of prostaglandins by inhibiting COX (190-192). Several phenolic compounds have been shown to decrease tumorigenesis through modulation of the arachidonic acid pathway (142, 193). Curcumin, quercetin, kaempferol, genistein, apigenin, resveratrol and resorcinol have all been shown to inhibit COX-2 activity and decrease formation of prostaglandins (2, 194). Resveratrol down-regulates the expression and activity of COX-2 in vitro

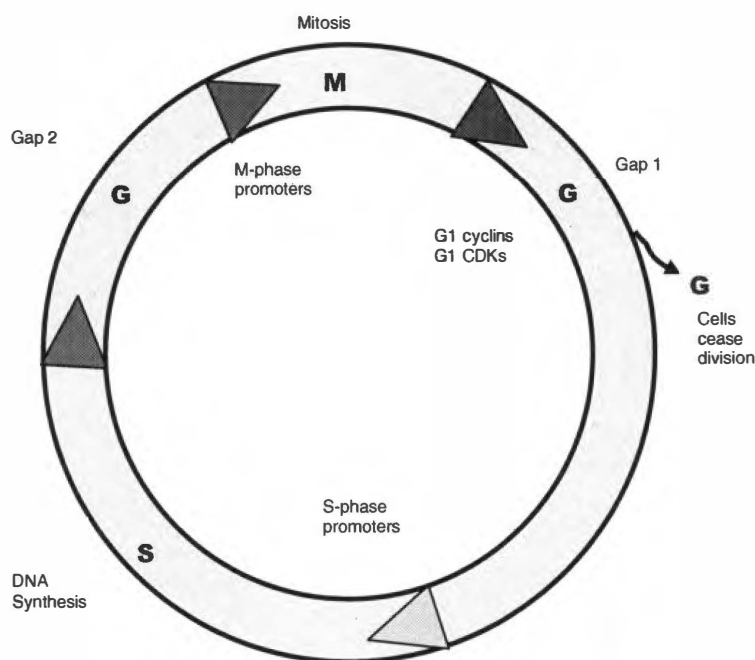


Figure I-5. The Cell Cycle

(190-191, 195). Resveratrol also inhibits the formation of prostaglandins by regulating the release of arachidonic acid from membrane phospholipids (196). Resveratrol may inhibit COX-2 expression and tumor cell growth by inhibiting the activation of $\text{NF}\kappa\beta$, a transcriptional regulator of COX-2 and other pro-tumorigenic factors. $\text{NF}\kappa\beta$ is a transcriptional regulator implicated in the activation of genes involved in intestinal cell migration, and proliferation and is normally sequestered in the cytoplasm by the $\text{I}\kappa\kappa$ complex consisting of $\text{I}\kappa\kappa\alpha$, $\text{I}\kappa\kappa\beta$, and $\text{NF}\kappa\beta$ -essential modulator (NEMO) (197). The $\text{TNF}\alpha$ signaling cascade leads to the phosphorylation of $\text{I}\kappa\kappa\alpha$, earmarking the $\text{I}\kappa\kappa$ complex for degradation in the proteosomes by the E3 ubiquitin ligase. Degradation of the $\text{I}\kappa\kappa$ complex results in the release and nuclear translocation of sequestered $\text{NF}\kappa\beta$ (Figure I-6,

p. 24), an action inhibited when degradation of the I κ κ complex is repressed.

This nuclear translocation results in the activation of target genes such as c-Jun, c-Fos, c-myc, Ras, ICAM, VCAM, COX-2, iNOS, which are involved in the tumorigenic process (198). The up-regulation of these factors culminates in decreased apoptosis, increased proliferation, and increased angiogenesis and overall, promotes tumorigenesis and tumor integrity.

In vitro evidence suggests that resveratrol inhibits proliferation and induces apoptosis of colorectal cancer cells by inhibiting NF κ β activation. In epithelial cells, resveratrol inhibits phosphorylation of I κ β , preventing degradation of the I κ κ complex and subsequent NF κ β translocation to the nucleus in a dose-dependent manner as evidenced by decreased nuclear content of NF κ β (199-200). These effects result in decreased NF κ β -dependent reporter gene transcription (201). Similar effects have been observed with other phytochemicals, such as capsaicin and curcumin, and curcumin and resveratrol blocked nuclear NF κ β -induced transcription of COX-2 by inhibiting the degradation of I κ β α (202).

As discussed previously, selective COX-2 inhibition impedes intestinal tumorigenesis by decreasing prostaglandin biosynthesis and prostaglandins are involved in the maintenance of tumor integrity by promoting angiogenesis and metastasis. Resveratrol and other polyphenolic phytochemicals with similar resorcin-containing structures have been shown to inhibit COX-2 and impede tumorigenesis by inhibiting the metastatic process in vitro and in vivo (203).

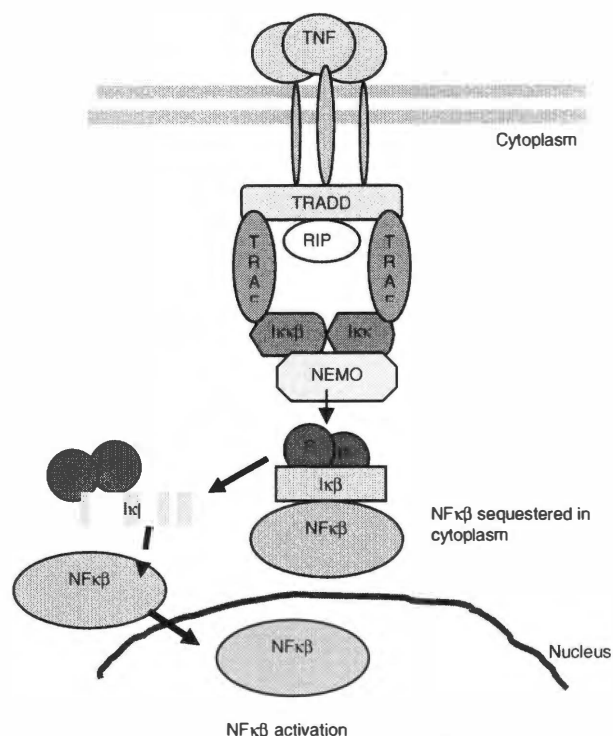


Figure I-6. The NFκβ Signaling Pathway

Resveratrol has been demonstrated to inhibit lung metastasis and capillary-like network formation (a measure of angiogenesis) in human umbilical vein endothelial cells (HUVECs) (204) and to decrease tumor volume, weight, and metastasis to lung in mice xenograft models of lewis lung carcinoma (205). In addition to these reports indicating metastatic inhibition, in vitro data in two breast cancer cell lines (MDA-MB-435, a highly invasive cell line and MCF-7, a non-invasive cell line) indicates that resveratrol may possess a higher affinity for those cells with greater invasive capacity (206-207).

Due to its polyphenolic ring structure, and similarity to diethylstilbesterol (DES) (**Figure I-7**, p. 25), resveratrol has been examined for a potential role as a phytoestrogen and has been shown to modulate mammary tumorigenesis. In

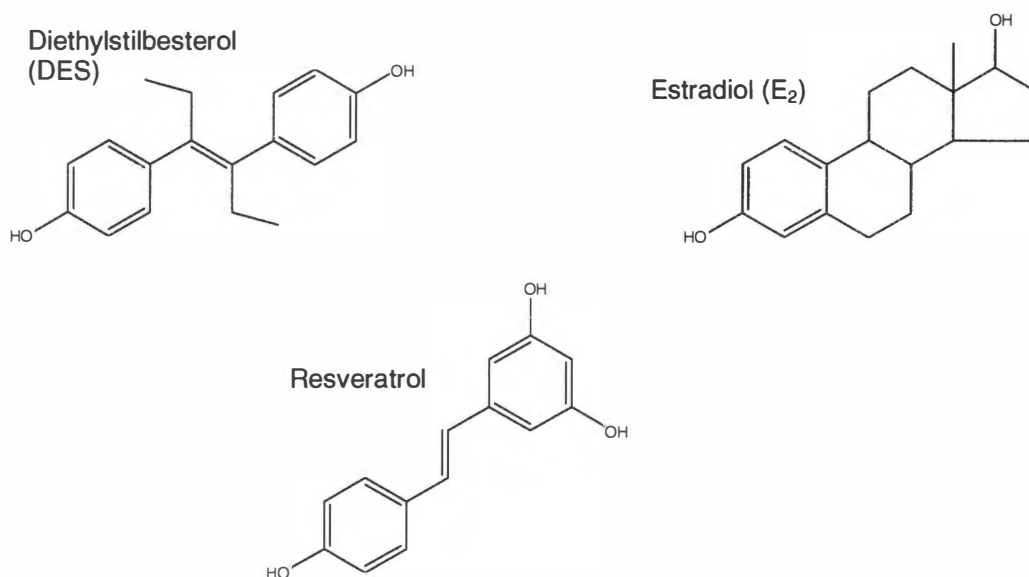


Figure I-7. Structures of Estrogenic Compounds

addition to its inhibition of COX-2 transcription and activity in human breast cancer cells (194), resveratrol has estrogen antagonist activity (208), and inhibits the proliferation of estrogen receptor-positive (ER+) MCF-7 breast cancer cells (208-209). However, in the same cell line, other reports indicate that resveratrol acts as an estrogen agonist, promoting cell growth (210-211).

The significance of its phytoestrogen activity in relation to colorectal cancer centers around the fact that the gastrointestinal tract expresses ER β and this expression is associated with estrogen-mediated modulation of intestinal cancers (212). In CHO-K1 breast cancer cells, resveratrol-bound ER β had increased transcriptional activity compared to estrogen-bound ER β , indicating that tissues, such as the intestine, that preferentially express ER β may be

sensitive to the estrogen agonist activity of resveratrol (213). However, the affinity of resveratrol for the ER is reported to be five orders of magnitude lower than that of DES or estrogen (E_2) (214). Therefore the role of resveratrol as an estrogen agonist via $ER\beta$ in colorectal cancers is most likely insignificant as compared to other proposed mechanisms.

Chapter II

Effects of resveratrol on intestinal tumor load, COX-2 expression and PGE₂ biosynthesis in a murine model of intestinal tumorigenesis

Abstract

To determine its effect on intestinal tumorigenesis and the pro-tumorigenic COX-2 pathway in *Apc^{Min/+}* mice, resveratrol was administered as a powdered admixture in the diet at 0, 4, 20, or 90 mg per Kg body weight for seven weeks. In two separate experiments, resveratrol had no effect on intestinal tumor load. Resveratrol was demonstrated to be stable in the diet under experimental conditions, circulated in the plasma in the glucuronide-conjugated form, and reached the tumors as evidenced by significant decreases in PGE₂ biosynthesis. However, immunohistochemical staining of intestinal tumors revealed no changes in COX-2 expression. This study demonstrates that resveratrol, as provided ad libitum in the diet, does not modify tumorigenesis in *Apc^{Min/+}* mice.

Introduction

Polyphenolic phytochemicals have been shown to be effective anti-tumorigenic agents in several in vitro models (203), and efficacy in vivo has also been demonstrated when these compounds are provided in the diet (148, 215-216). Resveratrol, a resorcinol-containing polyphenol found most commonly in red wine, inhibits the growth of several immortalized human colorectal cancer cell lines (217-218). When provided orally, resveratrol reduced tumor load in Lewis

lung carcinoma-bearing mice (219) and reduced the number of aberrant crypt foci in an azoxymethane (AOM)-induced rat model of colorectal cancer (220). Quercetin, curcumin, and caffeic acid phenyl ester, chemical analogs of resveratrol, inhibit intestinal tumorigenesis in vitro (221-222) and aberrant crypt formation in vivo (2, 84). These polyphenolic phytochemicals share a common resorcinol-containing structure, and this structure is believed to be partly responsible for the reported effects (2, 223).

Resveratrol's anti-tumorigenic effects have been linked, in part, to inhibition of cyclooxygenase (COX) catalyzed reactions (190, 192, 224-225). COX catalyzes the committed step in the conversion of arachidonic acid to pro-tumorigenic eicosanoids, such as PGE₂, which are involved in the maintenance of tumor integrity (125). COX-2 expression is up-regulated in 80-85% of human adenocarcinomas and colonic tumors (100, 226-227) and in 80-85% in *Apc*^{Min/+} of mouse adenomas (228). Resveratrol has been shown to inhibit COX-2 promoter activity (224), down-regulate COX-2 expression (192, 224) and inhibit COX-2 activity (190, 229) in vitro.

Numerous studies have demonstrated the ability of COX inhibitors to decrease tumor multiplicity in the *Apc*^{Min/+} mouse, an animal model possessing a germline mutation in the *adenomatous polyposis coli* (*Apc*) tumor-suppressor gene (230-233). These mice spontaneously develop an average of forty tumors throughout the intestinal tract that are sensitive to COX inhibition. Murine *Apc* is homologous to human *APC* (75), and patients with familial adenomatous polyposis (FAP) have an autosomal dominant disorder with a germline mutation

in one allele of the *APC* tumor suppressor gene (27). Adenoma formation results when a somatic mutation is acquired in the remaining functional allele. By the time they reach their twenties, FAP patients typically develop hundreds to thousands of intestinal adenomas, mostly in the colorectal mucosa, which are also sensitive to COX inhibition (234). Similarly, at least one allele of *APC* is mutated in approximately 80% of sporadic colorectal cancers, making the *Apc*^{Min/+} mouse a relevant model for studying the early stages of colorectal cancer (66, 235-236).

The purpose of this study was to evaluate the dose-dependent effects of dietary resveratrol on intestinal tumorigenesis in the *Apc*^{Min/+} mouse, and to determine whether tumor COX-2 levels and PGE₂ biosynthesis are affected by this treatment.

Materials and Methods

Animals

Male C57BL/6J *Apc*^{Min/+} mice or wild type littermates (Jackson Laboratories, Bar Harbor, ME, USA) were obtained following genotyping at 35 days of age and randomly assigned to their respective experimental groups. Animals were provided water and diet ad libitum. Diet intake and overall health was monitored daily and body weights were monitored weekly. All animal research procedures were approved by the University of Tennessee Animal Care and Use Committee and were in accordance with the NIH Guide for the Care and Use of Laboratory Animals (237).

Diets

AIN-93G powdered diet (Dyets, Inc., Bethlehem, PA, USA) was used as the base diet. Experimental diets were prepared weekly by thoroughly mixing resveratrol (Sigma Chemical Co., St. Louis, MO, USA) with the base diet to homogeneity. Resveratrol concentrations in the diets were adjusted weekly, based on food consumption and body weights to achieve doses of 0, 4, 20, and 90 mg per Kg body weight. Diets were prepared under yellow light and stored at -80°C under an atmosphere of nitrogen to minimize degradation of resveratrol. Fresh diet was provided daily.

Experimental Design

Pharmacokinetics and absorption of resveratrol

To establish the stability of resveratrol in the diet, dietary samples containing resveratrol at 750 ppm (dose equivalent to the animals fed 90 mg per Kg body weight) (n=2) were stored under an atmosphere of nitrogen at -80°C (controls), or exposed to experimental conditions (light and room temperature for twenty-four hours). Samples were extracted with ethyl acetate (x4) (Sigma), evaporated under an atmosphere of nitrogen, re-suspended in the reversed-phase high performance liquid chromatography (RP-HPLC) mobile phase and analyzed for resveratrol content via RP-HPLC.

To establish that resveratrol reaches the intestinal tract intact and is subsequently absorbed, fifteen 10-week-old male C57BL/6J mice were provided resveratrol in the diet at a dose of 200 mg per Kg body weight. Before sacrifice, the animals were divided into five groups (n=3/group). After six days on the diet, blood was collected post-prandially via cardiac puncture (under general anaesthesia) at 5, 15, 30, 60 and 90 minutes, and plasma resveratrol content was analyzed via RP-HPLC. In a follow-up experiment, three 10-week-old C57BL/6J wild type mice were fed resveratrol at 90 mg per Kg body weight. After six days of feeding, blood was collected 30 minutes post-prandially and samples were analyzed for resveratrol content via RP-HPLC.

Effect of resveratrol on intestinal tumorigenesis in the *Apc^{Min/+}* mouse model

Twenty-five male C57BL/6J *Apc^{Min/+}* mice (43 days of age) were assigned to four groups (6-7 mice per group) and fed diets supplemented with resveratrol at 0, 4, 20 and 90 mg per Kg body weight for seven weeks, until 85 days of age. Tumor number, size, and location were determined with the use of a dissecting microscope. Whole blood was collected via cardiac puncture, and immediately placed on ice protected from light. Plasma was isolated and stored at -80°C for analysis of resveratrol via RP-HPLC.

Effects of Resveratrol on COX-2 levels and PGE₂ biosynthesis in tumors

To establish the effect of resveratrol on COX-2 levels and PGE₂ biosynthesis in tumors, 16 male C57BL/6J *Apc*^{Min/+} mice were assigned to a control or experimental group (n=8) and fed resveratrol at a dose of 0 or 90 mg per Kg body weight for seven weeks. At 87 days of age, animals were sacrificed and excised intestinal tumors were analyzed for COX-2 expression and basal PGE₂ levels.

RP-HPLC analysis of trans-resveratrol

Resveratrol levels were analyzed by RP-HPLC using a Hewlett Packard 1090 Series-2 Liquid Chromatograph (Hewlett Packard, Palo Alto, CA, USA) equipped with a 25 cm, 4.6mm KR100-5C18 Kromasil RP-HPLC column (Akzo Nobel - EKA Chemicals, Bohus, Sweden) with an isocratic mobile phase consisting of 70% CH₃CN, 30% MeOH, 0.1% glacial acetic acid (Sigma), at a flow rate of 0.5 ml/min. Samples were monitored at 306 nanometers (nm) using a photodiode array detector. Maximum absorption of resveratrol is reported at 306nm (238). Detection and spectrophotometric identification limits were 2ng and 3ng, respectively.

Extraction of resveratrol from plasma

To determine relative levels of free versus glucuronidated resveratrol in the plasma, whole blood was collected via cardiac puncture, using ethylenediaminetetraacetic acid (EDTA), (Life Technologies, Grand Island, NY,

USA) at a concentration of 7.5% as an anti-coagulant, and immediately placed on ice, protected from light. Plasma was isolated by centrifugation at 500 x g, 4°C for 15 minutes. To determine relative levels of resveratrol circulating in the free form, 200 µl of plasma were aliquoted into an eppendorf tube, diluted 1:10 with MeOH, adjusted to a pH of 3.5 with formic acid, and samples were subjected to solid-phase extraction using a conditioned C18 cartridge (Burdick and Jackson, MI, USA). Resveratrol was eluted from the column (x2) with 2 ml of 100% HPLC-grade methanol (Sigma), evaporated under an atmosphere of nitrogen, re-suspended in the RP-HPLC mobile phase, and analyzed via RP-HPLC.

To determine relative levels of resveratrol circulating as the glucuronide derivative, 200 µL of plasma were aliquoted into an eppendorf tube for enzymatic hydrolysis via β-glucuronidase. Powdered H-1 β-glucuronidase (Sigma, St Louis, MO, USA) was solubilized at 50 mg/mL in 0.2 mol/L sodium acetate buffer, pH 5.0 at 37°C. This enzyme solution was added to plasma at 5:1, to a final concentration of 0.04 g/ml, and final specific activity of 5×10^3 µg/min/mg protein. Samples were incubated for 2 hours at 37°C in a rocking incubator. The reaction was terminated with 100% methanol, pH 3.0 with formic acid, to a final volume of 10% MeOH. Samples were extracted (x3) with equal volumes of ethyl acetate (Sigma) and pooled organic phases were evaporated under nitrogen, re-suspended in 1 ml 10% aqueous methanol solution, and passed thru a 0.45µm filter (Pall Corporation, Ann Arbor, MI, USA). Resveratrol was isolated via solid phase extraction as described previously and analyzed via RP-HPLC.

Immunohistochemistry for COX-2 expression

This analysis was conducted by Leah Rainwater. The tissues were formalin-fixed, embedded in paraffin, and sectioned at 4 μ M. Tissue sections were then heated, de-paraffinized in xylene, re-hydrated in graded alcohols to phosphate buffered saline (PBS) (3x, 5 minutes each), and pre-treated at or just below boiling with 10 mmol/L citrate buffer, pH 6, for 10 minutes. Endogenous peroxidase activity was quenched by incubation in 3% H₂O₂ PBS for 15 minutes at room temperature and incubated for 30 minutes at room temperature with protein block (Biogenex, San Ransom, CA). Slides were then incubated at 20°C for 1 hour with affinity-purified rabbit polyclonal anti-mouse COX-2 IgG (1:200) (Cayman Chemical), followed by biotinylated anti-rabbit IgG (30 minutes at room temperature) and streptavidin/biotin-horseradish peroxidase complex (30 minutes at room temperature) which was localized by reaction with 3,3'-diaminobenzidine tetra-hydrochloride (Biogenex) (0.7 mg/ml) and 0.05% H₂O₂ in 80mmol/L Tris-HCl, pH 7.6, for 10 minutes. Both positive and negative slides were lightly counterstained with Mayer's hematoxylin. Staining for COX-2 protein was evaluated from coded slides and scored blindly by a pathologist based on distribution (extent) and relative staining intensity. Relative staining distribution was scored as follows:

0 = < 10 stained cells per high power field (0.2 mm² at 400x magnification)

1 = 10-20 stained cells per high power field

2 = 20-30 stained cells per high power field

3 = > 30 stained cells per high power field

Both positive and negative tumors (i.e. distribution = 0) were included in calculating mean scores for each group. Relative staining intensities were graded as follows:

0 no staining

1 representing perinuclear staining

2 representing stain extending to the cytoplasm

3 indicating a stain which obscured the cell nucleus

Intensity and distribution scores were then added to generate a final value corresponding to COX-2 expression. The reported staining scores represent the sum of intensity and distribution $\pm$ SEM.

Basal PGE₂ Levels

To determine the effect of resveratrol on PGE₂ levels in tumors, intestinal tumors from *Apc*^{Min/+} mice fed resveratrol in the diet at 0 or 90 mg per Kg body weight resveratrol in the diet were excised on dry ice, snap frozen in liquid nitrogen, and stored at -80°C. At the time of analysis, tumors were pooled from each animal and homogenized using a Polytron handheld homogenizer on ice at 10:1 in ice-cold 0.1 mol/L Tris-HCl buffer, pH 7.4. An aliquot of 50 μ l from each group was used for protein determination via the Bradford assay (246). PGE₂ levels were determined by enzyme immunoassay (EIA) using the Correlate-EIA PGE₂ Kit (catalog # 900-001), (Assay Designs) according to the manufacturer's instructions.

Statistical analyses

Statistical analysis was carried out using the Statistical Analysis System and JMP software (SAS Institute, Inc, Cary, NC, USA). Data was checked for normality of distribution and homogeneity among variances. Differences in body weight, diet intake, tumor number and basal PGE₂ levels were analyzed by student's t-test or one-way ANOVA. Differences among groups were detected using Fisher's least significant difference at $P < 0.05$. Data are expressed as means $\pm$ SEM.

Results

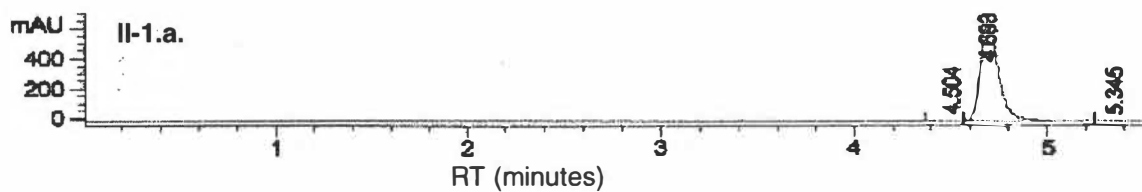
No significant differences in food intake were observed between any of the treatment groups. During the last week of treatment, animal weights in the control group were significantly lower when compared to the low-dose treatment group (4 mg per Kg body weight). No other groups were significantly different.

To determine the general stability characteristics of resveratrol when exposed to ultra-violet (UV) light, and to identify the absorption spectra of UV degradation products of resveratrol, a solution containing resveratrol solubilized in the RP-HPLC mobile phase was analyzed by RP-HPLC, revealing a retention time of 4.693 minutes (**Figure II-1a**, p. 38) and the standard UV absorption spectra of resveratrol (**Figure II-1b**, p. 38). This solution was then exposed to constant UV light for 18 days and analyzed via RP-HPLC every 2 days for its degradation products. Resveratrol was stable up to 14 days. After 14 days, two additional peaks were observed at 4.578 and 5.228 minutes (**Figure II-2 a**, p. 39),

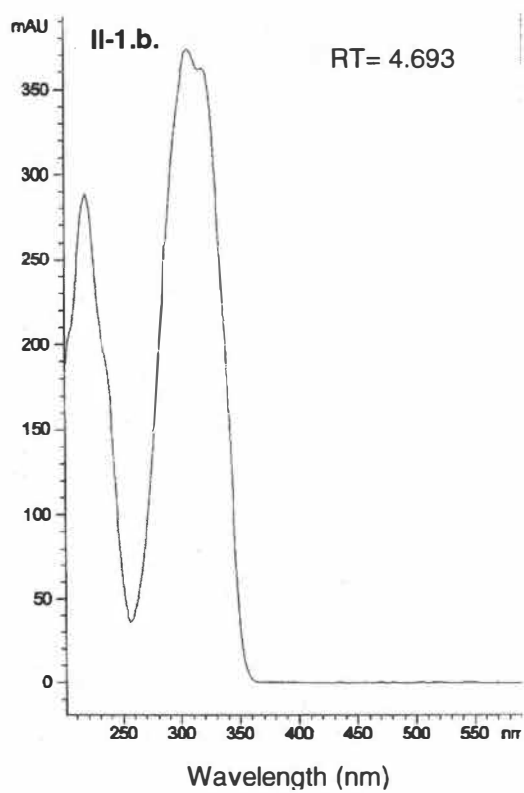
corresponding to two resveratrol isomerization products (240) identifiable by distinct UV absorption spectra (**Figure II-2.b**, p. 39, **Figure II-2.c**, p.40). No isomerization products of resveratrol were detected in the diet when it was exposed to the same conditions present in the feeding study (**Figure II-3.a**, p. 41, **Figure II-3.b**, p. 41, **Figure II-3.c**, p. 42, **Figure II-3.d**, p. 42), suggesting that resveratrol remained stable in the diets under experimental conditions (**Table II-1**, p. 50).

When non-hydrolyzed plasma of animals fed resveratrol at 200 mg per Kg body weight was analyzed, the RP-HPLC chromatogram reveals a peak at 4.74 minutes (**Figure II-4.a**, p.43), however no UV absorption spectrum of resveratrol was identifiable at that retention time (**Figure II-4.b**, p. 43). Resveratrol was detected in the plasma of animals following hydrolysis of the glucuronide derivative at 200 mg per Kg body weight at 4.720 minutes (**Figure II-4.c**, p. 44, **Figure II-4.d**, p. 44) and at 90 mg per Kg body weight at 4.661 minutes (**Figure II-4.e**, p. 45, **Figure II-4.f**, p. 45). No resveratrol was detected in the plasma of animals when not provided orally. Pharmacokinetic data from hydrolyzed plasma revealed that plasma resveratrol was detected within 5 minutes, peaked by 30 minutes and was maintained for at least 90 minutes, post-prandially (**Figure II-5**, p. 46).

In comparison to untreated controls, resveratrol had no effect on intestinal tumor load when provided in the diets of *APC^{Min/+}* mice from 4-90 mg per Kg body weight (**Figure II-6**, p. 47). A second experiment yielded similar results, with no significant difference in tumor load observed between animals fed 0 and 90 mg

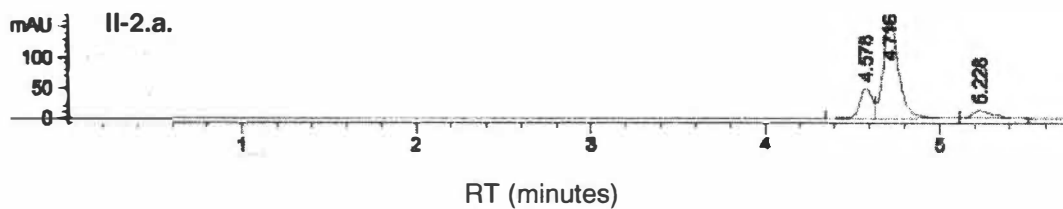


a. RP-HPLC chromatogram of resveratrol standard in mobile phase.

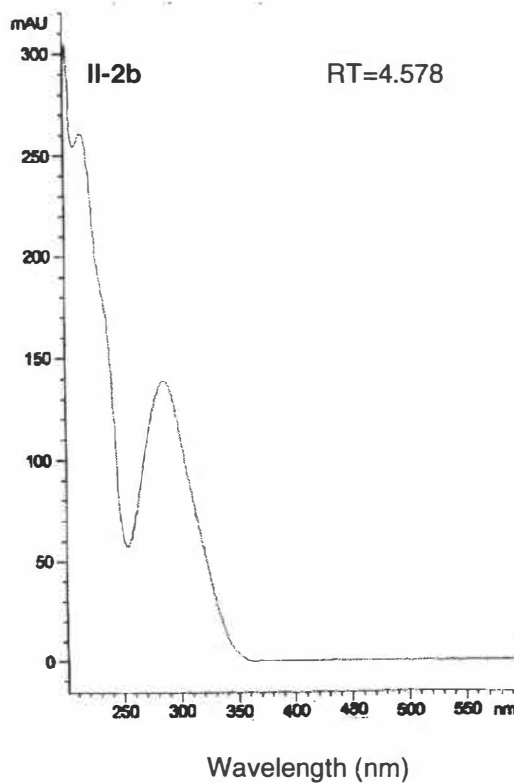


b. RP-HPLC UV absorption spectra of peak from chromatogram 1a. RT=4.693.

Figure II-1. Resveratrol Standard

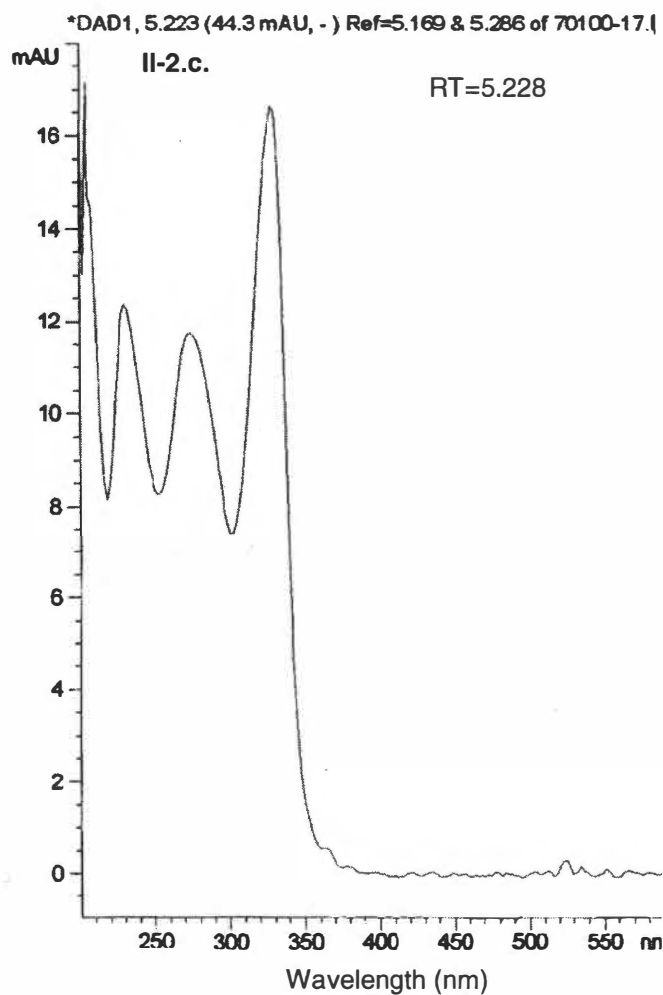


a. RP-HPLC chromatogram of resveratrol isomerization products derived after 2 weeks of continuous sunlight exposure.



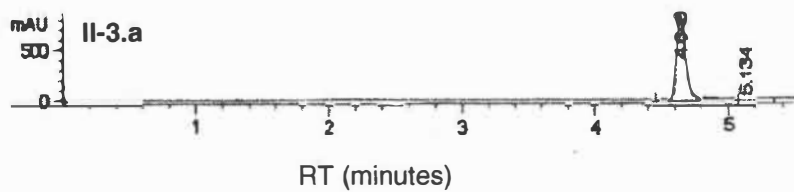
b. RP-HPLC UV absorption spectra of peak from chromatogram 2a. RT=4.578

Figure II-2. Resveratrol UV Degradation Products

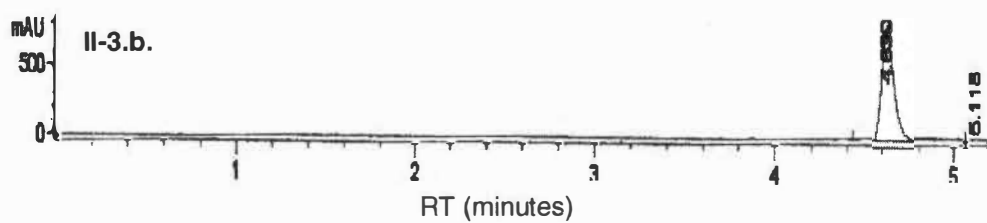


c. RP-HPLC UV absorption spectra of peak from chromatogram 2a. RT=5.228.

Fig. 11-2. continued

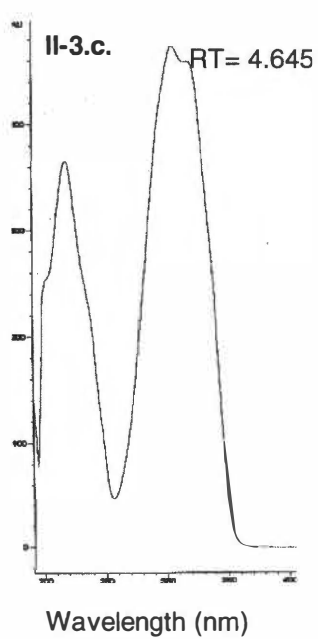


a. RP-HPLC chromatogram of resveratrol extracted from control diet.

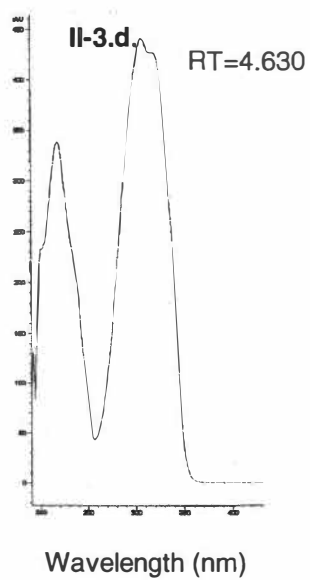


b. RP-HPLC chromatogram of resveratrol extracted from experimental diet.

Figure II-3. Resveratrol Stability in the Diet

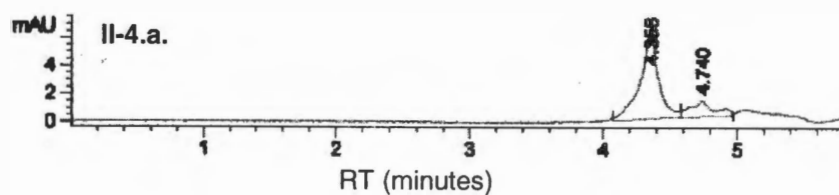


c. RP-HPLC UV absorption spectra of peak from chromatogram 3a. RT=4.645.

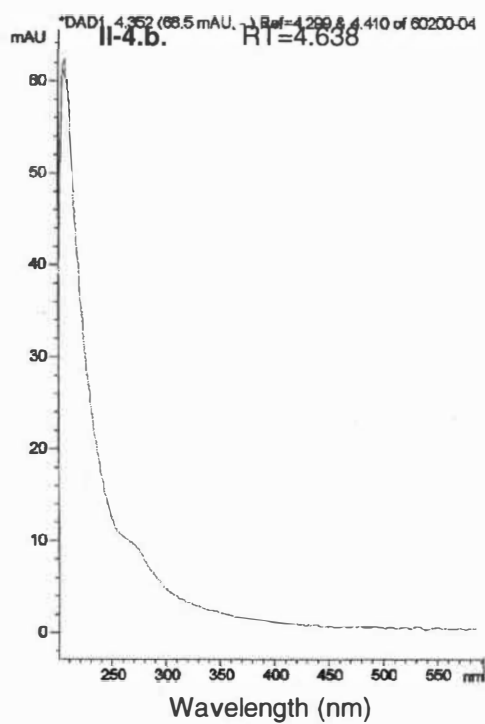


d. RP-HPLC UV absorption spectra of peak from chromatogram 3b. RT=4.630

Fig. II-3. continued

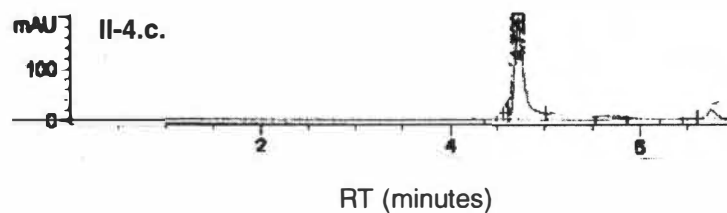


a. RP-HPLC chromatogram of non-hydrolyzed plasma extracted from animal fed resveratrol at 200 mg/Kg BW.

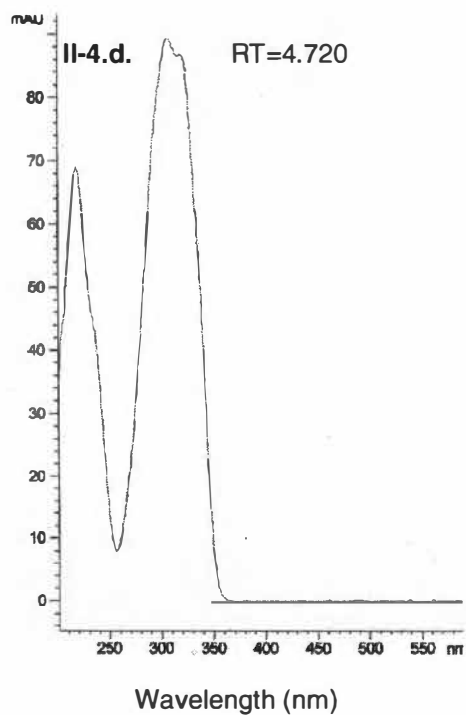


b. RP-HPLC UV absorption spectra of peak from chromatogram 4a. RT=4.740

Figure II-4. Resveratrol Pharmacokinetics

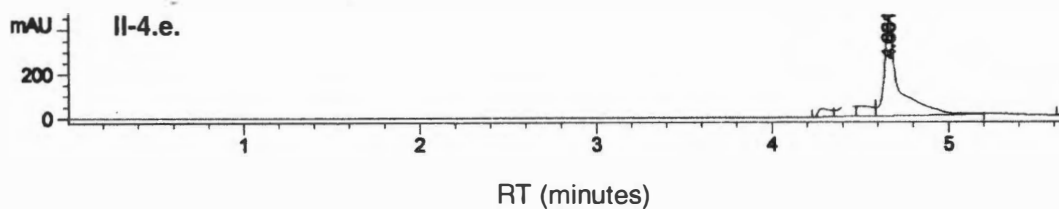


c. RP-HPLC chromatogram of hydrolyzed resveratrol extracted from plasma of animal fed 200 mg per Kg BW resveratrol, 30 minutes post-prandial time.

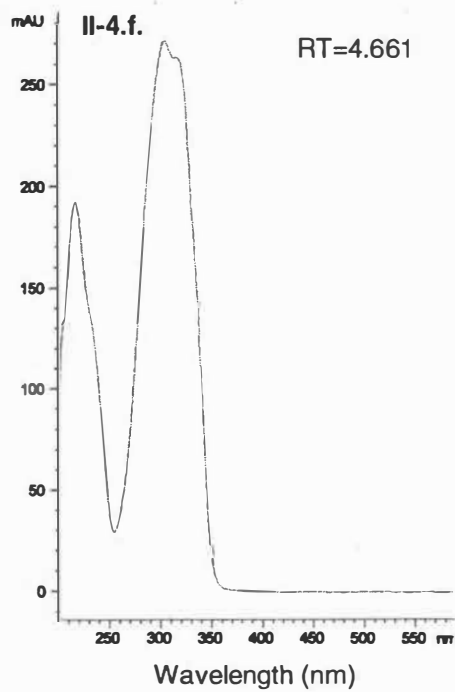


d. RP-HPLC UV absorption spectra of peak from chromatogram 4c. RT=4.720

Fig. II-4. continued



e. RP-HPLC chromatogram of hydrolyzed resveratrol extracted from plasma of animal fed 90 mg per Kg BW, 30 minutes post-prandial time.



f. RP-HPLC UV absorption spectra of peak from chromatogram 4e. RT=4.661

Fig. II-4. continued

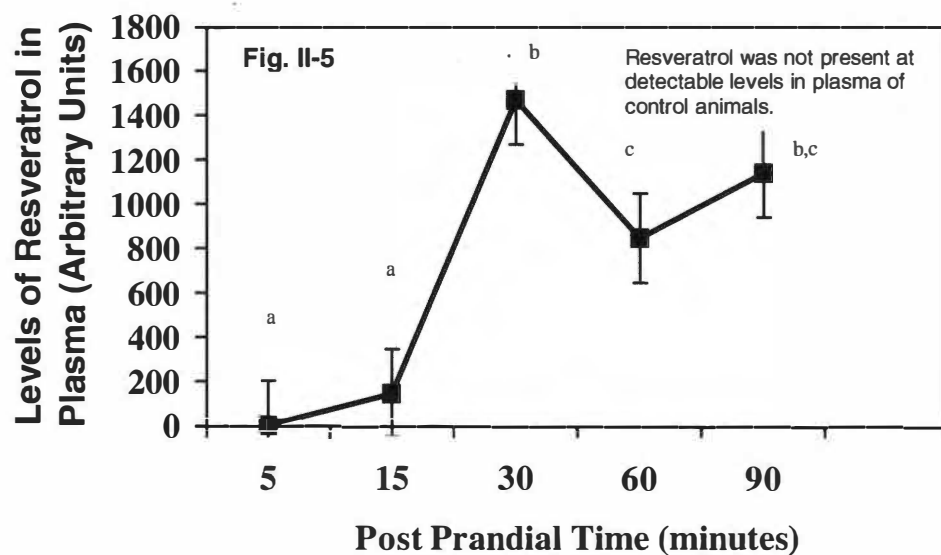


Figure II-5. Effect of post-prandial time (5, 15, 30, 60, 90 minutes) on plasma resveratrol levels after administration of 200 mg per Kg BW dose as compared to controls (0 mg per Kg BW dose).

Plasma was collected and analyzed as described in materials and methods.

Error bars indicate $\pm$ SEM, $n=3$. Different superscripts indicate statistical significance at $p < 0.05$.

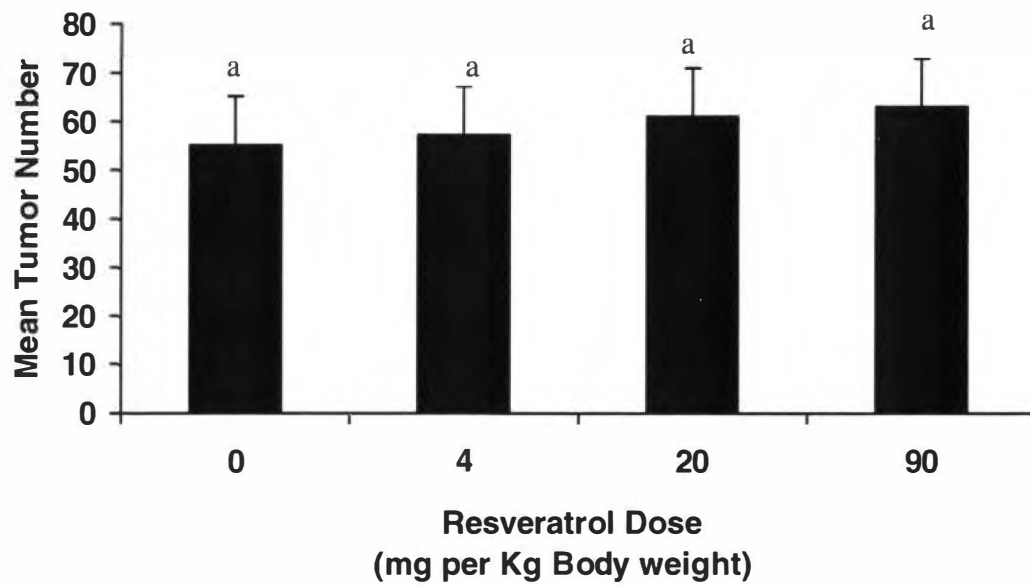


Figure II-6. Effect of dietary resveratrol on intestinal tumor number in *APC^{Min/+}* mice.

Resveratrol was administered to animals at 0-90 mg per Kg BW. Tumors were analyzed as described in materials and methods. Values are means $\pm$ SEM, n=6-7. Different superscripts indicate differences among groups at $p < 0.05$.

resveratrol per Kg body weight (**Table II-2**, p. 51). Basal PGE₂ levels in tumors of animals fed resveratrol at 90 mg per Kg body weight were measured by EIA as an index of relative COX activity compared to the control animals (0 mg resveratrol per Kg body weight). PGE₂ levels of intestinal tumors were significantly lower in the resveratrol-treated animals as compared to controls (**Table II-2**). There was no significant difference in COX-2 protein levels between the control animals (mean score = 0.69 ± 0.20) and animals fed resveratrol at 90 mg per Kg body weight (mean score = 1.09 ± 0.24) (**Figure II-7**, p. 49). COX-2 localization was constrained to the stromal cells of the tumor with no detectable levels in the epithelium. This confinement of COX-2 expression to stromal cells as opposed to neoplastic epithelium has been reported previously (113) and suggests that prostaglandins may exert their pro-tumorigenic effects via paracrine signaling.

Discussion

Resveratrol's chemotherapeutic effects in vitro are believed to be related, in part, to inhibition of COX-2 expression and activity (190), and these same biochemical pathways are linked to intestinal tumorigenesis in *Apc^{Min/+}* mice (125, 230, 241). However, we report that resveratrol (0-90 mg per Kg body weight) failed to decrease the number of intestinal tumors following seven weeks of treatment.

Analysis of the diets revealed that resveratrol remained stable during the time of feeding, and plasma pharmacokinetic data indicated adequate

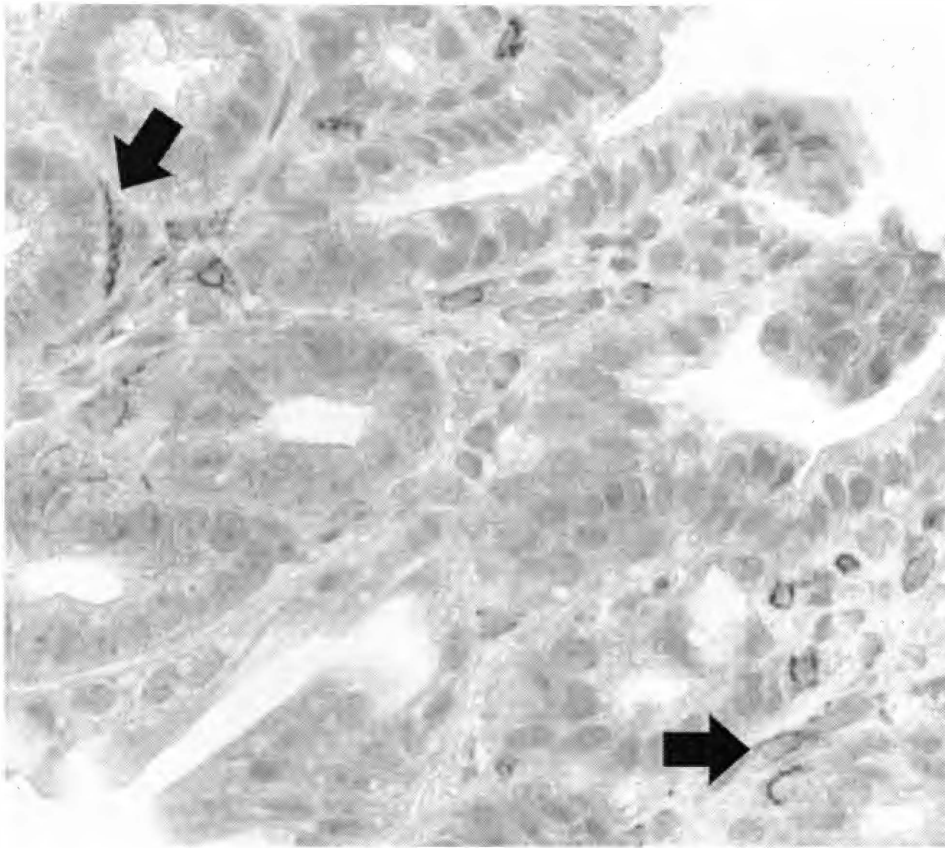


Figure II-7. Effect of dietary resveratrol on COX-2 expression in intestinal tumors of APC^{Min/+} mice.

Representative photomicrograph of small intestinal tumor immunohistochemically stained for COX-2 enzyme expression in an animal treated with resveratrol. Arrows indicate positive (brown) COX-2 expression in stromal cells. Original magnification 400X.

Table II-1. Stability of resveratrol in animal diets under 24- hour experimental conditions as compared to non-exposed controls

	Control Diet	Exposed Diet
% Control	100 ^a	96 ^a

Resveratrol was extracted from experimental and control diets and measured via RP-HPLC as described in materials and methods. Values are expressed as percent of control. ^a There was no significant difference in levels of trans-resveratrol between control and exposed diets at $p < 0.05$.

Table II-2. Effect of dietary resveratrol at 90 vs 0 mg per Kg BW on tumor number and tumor PGE₂ biosynthesis

	Resveratrol dose in mg/Kg BW	
	0	90
Mean tumor #	32.86 ± 5.56 ^a	25.86 ± 5.88 ^a
pg PGE ₂ /mg protein	1.32 ± 0.62 ^a	0.78 ± 0.62 ^b

¹Intestinal tumors were analyzed for PGE₂ levels as described in materials and methods. Values are expressed as means ± SEM. Different superscripts in each row indicate statistically significant differences among groups at p<0.05.

absorption. Resveratrol and other polyphenols are absorbed in the small intestines where they undergo glucuronidation before appearing in the blood (242). Our data demonstrate that 100% of resveratrol exists as the glucuronide conjugate in the circulation. However, there is much debate as to whether this form of resveratrol is biologically active. In vitro studies utilize the free form of resveratrol and derivatization in vivo could result in a less biologically active compound. Furthermore, studies in vivo are lacking as to whether there is adequate β -glucuronidase activity to generate sufficient levels of free resveratrol in vivo to mimic the in vitro results. We observed no measurable levels of free resveratrol in the plasma of resveratrol-treated mice, and resveratrol was only detected following enzymatic hydrolysis, consistent with complete glucuronidation. Human and rat small intestines have sufficient β -glucuronidase activity to hydrolyze naturally occurring glucuronide derivatives of polyphenols found in the diet, but it does not appear that this activity is sustained within the circulation (243-245). Resorcin-containing polyphenols such as resveratrol, quercetin, and catechins display similar pharmacokinetics to the kinetics observed here, with peak plasma levels occurring at approximately $\frac{1}{2}$ -1 hour post-ingestion, and others have confirmed that greater than 95% of all of these compounds circulate in the plasma in their conjugated forms (139, 246-248).

We failed to observe any reduction in tumor load with dietary levels of resveratrol that ranged from 4 to 90 mg per Kg body weight. These results are in contrast to a previous paper reporting a 74% reduction in tumor frequency in this

model when a comparable dose of resveratrol was provided in the drinking water dissolved in ethanol (249). While ethanol may increase β -glucuronidase activity in tissues such as liver and lung (250-252), it appears that the presence of ethanol as a vehicle used in the oral administration of polyphenols, does not increase the ability of the intestinal tract to hydrolyze glucuronide derivatives (139). Therefore, providing resveratrol in the diet or drinking water dissolved in ethanol should not increase circulating levels of the free form of the compound and cannot explain the disparate results observed between the two studies. In an effort to duplicate our results, we repeated the study using the highest dose (90 mg per Kg body weight), and again observed no significant decreases in tumor load with dietary resveratrol.

Furthermore, our results confirm that resveratrol was absorbed and reached the target tissue as evidenced by reductions in PGE₂ biosynthesis in tumors of resveratrol-fed animals as compared to controls. The doses administered are comparable to doses administered in a previous study (249) and the length of the study (seven weeks) should have been adequate to observe any decreases in tumor load. Our estimated concentration of 131uM/L for total plasma resveratrol (90 mg per Kg body weight group) far exceeds the plasma levels (1.5uM/L) from a volunteer given a 25 mg oral dose (~0.4 mg per Kg body weight for a 70 Kg individual) (244). To fully appreciate these doses, the amount of resveratrol in a liter of table wine varies from 0.1-7.7 mg (240). Most wines, however, contain concentrations that are less than 0.5 mg/L (240).

Discordant effects on resveratrol's ability to modify tumorigenesis have been reported elsewhere, particularly when comparing results in vitro versus in vivo. Resveratrol inhibited the in vitro growth of 32Dp210 mouse myeloid leukemia cells and resveratrol-pretreated cells implanted subcutaneously; however, when the same cell line was implanted subcutaneously without pretreatment and mice were given resveratrol orally, no inhibition of growth was observed (4). Similarly, orally-administered resveratrol failed to inhibit tumorigenesis in a chemically-induced lung tumor model (253). Yet, resveratrol has been shown to inhibit tumorigenesis in a carcinogen-induced rat model of colon cancer (220), Lewis lung carcinoma-bearing mice (254), and in a DMBA-induced mammary tumor mouse model (229). Similarly, dichotomous results have been reported with other structurally related polyphenolic compounds. For example, quercetin was unable to reduce the number of tumors in *Apc*^{Min/+} mice, but effectively reduced tumor load in a murine model of carcinogen-induced colon cancer (147). Likewise, genistein inhibited the growth of MDA-MB-231 cells (255) in vitro, but was ineffective when the cells were implanted in mice that were given genistein orally (256). These disparate results make it difficult to predict the in vivo effects of this compound at human equivalent doses.

We investigated the effect of resveratrol on PGE₂ biosynthesis and COX-2 expression in intestinal tumors because we, and others, have established that COX inhibition reduced the growth of intestinal tumors and regressed established tumors in this animal model as reviewed by Whelan and McEntee (257). Chemopreventive agents containing a common resorcin moiety (e.g., resveratrol,

quercetin, genistein) suppressed COX-2 transcription and activity in a human colon cancer cell line (224) and selective inhibition of COX-2 inhibited tumorigenesis in *Apc^{Min/+}* mice (258). Resveratrol inhibited COX-2 transcription and directly inhibited COX-2 activity in vitro (192, 259). We failed to observe any difference in COX-2 expression as a result of resveratrol treatment. However, we did detect PGE₂ levels that were 41% lower in tumors from the resveratrol-fed animals, but this change was apparently insufficient to impact tumor number. Inhibition of PGE₂, viewed as an isolated event, is not always associated with a reduction in tumors (241, 260).

Summary and Conclusions

In summary, resveratrol was shown to be stable in the diet, was absorbed intact and reached the target tissue, but had no effect on intestinal tumorigenesis at 3 doses and in two independent experiments. These results may be explained, in part, by the fact that 1) resveratrol was ineffective as a modulator of COX-2 expression in tumors, 2) the decreases in PGE₂ formation were insufficient to modify tumor integrity, and/or 3) the levels of free resveratrol did not attain a sufficient concentration to duplicate the antitumorigenic effects observed in vitro. Further experimentation is required to investigate the seemingly inconsistent effects of resveratrol using in vivo versus in vitro models.

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APPENDIX

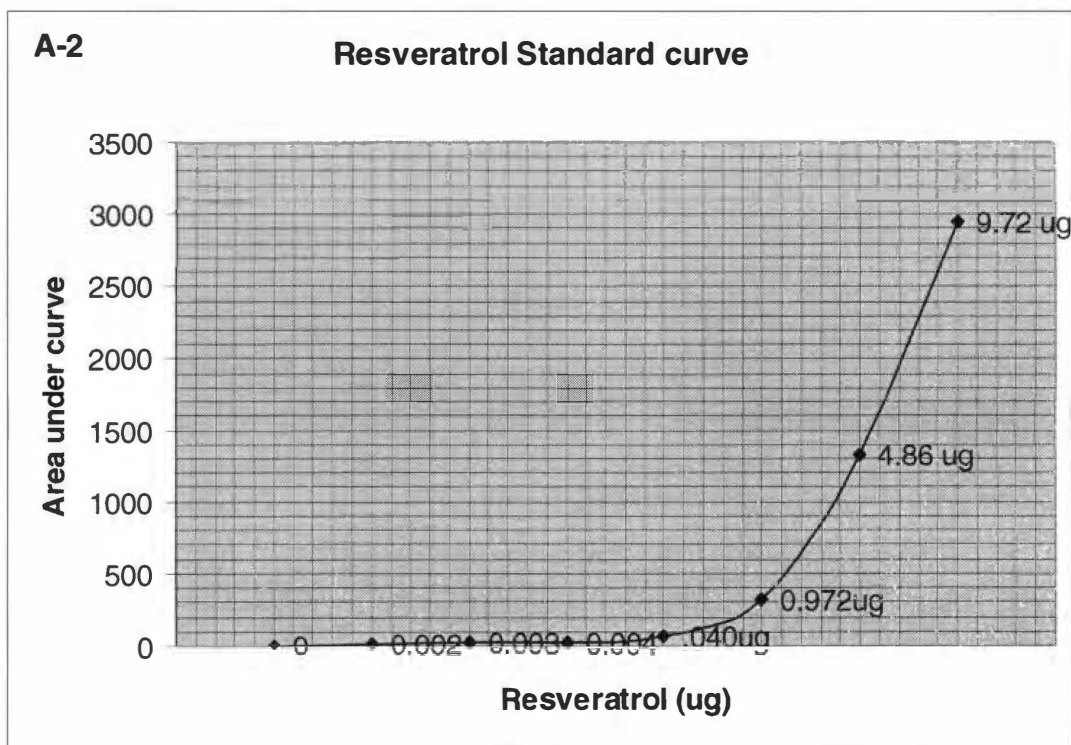
APPENDIX

A-1

Ingredient	g/Kg	Kcal/g	Kcal/Kg
Casein	200	3.58	716
Cornstarch	397.486	3.6	1430.95
Dyetrose	132	3.8	501.6
Sucrose	100	4	400
Cellulose	50		
Soybean Oil	70	9	630
t-Butylhydroquinone	0.014		
Salt mix# 210025	35	0.88	30.8
Vit mix# 310025	10	3.87	38.7
L-Cystine	3	4	12
Choline Bitartrate	2.5		

Total **3760.05**

A-1. Nutrient Composition of AIN-93G diets



A-2. RP-HPLC Standard Curve for Resveratrol Quantitation

VITA

Carol Cathleen Ziegler was born on August 19, 1976 in Nashville, Tennessee. She completed her undergraduate training in Nutrition at the University of Tennessee in May of 1999, and went on to complete a Masters thesis in the same field in December, 2002. She will pursue a doctorate degree in the near future and hopes to continue working in the area of nutrition and cancer research.