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Assessing the use of piceid (resveratrol-glucoside)-accumulating transgenic alfalfa against colon cancer

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TABLE OF CONTENTS

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ABSTRACT

Biotechnological approaches have been applied to introduce *resveratrol-synthase (RS)* (aka. *Stilbene synthase*) into novel crops such as apple, kiwi, tomato and wheat. As with natural sources of resveratrol, genetically-modified plants, which constitutively express RS, accumulate resveratrol primarily in the form of piceid (RG). The chemopreventive activity of a RG-accumulating transgenic alfalfa (variety Regen SY) against colon cancer was assessed in an azoxymethane (AOM)-induced aberrant crypt foci (ACF) rodent model of carcinogenesis. Our initial studies revealed that the transgenic alfalfa (TA) at 20% in the diet by weight had no affect on the number, size or multiplicity of ACF in CF-1 mice after a five-week feeding trial. However, the number of AOM-induced ACF was reduced in mice fed these diets supplemented with a β-glucosidase enzyme relative to mice fed TA alone. To test that RG is not bioavailable to the colon, the colonic concentrations of resveratrol-aglycone (Rag) and RG were measured following five-week administration of the diets. Rag, but not RG, was detected by liquid chromatography/mass spectrometry in the colons of mice fed transgenic alfalfa, but these concentrations tended to be lower in mice fed TA without α-galactosidase relative to mice fed these diets supplemented with enzyme. The use of a lactase inhibitor in transport studies conducted in everted, jejunal sacs from CF-1 mice suggested that RG was a substrate for lactase-phlorizin hydrolase and can be absorbed intact in the intestines, also. However, comparative studies with Rag suggest that the intestinal absorption of the Rag was much more efficient than that of RG. Rag could be detected in the plasma and the epithelia of the colon and small intestine of CF-1 mice intragastrically fed 25 mg (111 μM) Rag/kg body weight 1-7 hours post-gavage, but neither Rag nor RG could be detected in mice fed an equal dose of piceid at these time points. Collectively, these data

suggest that the intestinal bioavailability of RG was much less than that of Rag. The findings of these studies raise doubt on the ability to achieve the purported health benefits of resveratrol through natural sources alone where resveratrol is present as RG.

CHAPTER 1: GENERAL INTRODUCTION

Introduction

Resveratrol (3,5,4'-trihydroxystilbene) is a polyphenolic phytochemical that is structurally and functionally related to flavonoid compounds, and is produced by a variety of plant species as a secondary metabolite (Fremont, 2000). A number of studies conducted in cell culture systems and animal models suggest that resveratrol may possess a multitude of health benefiting properties and may offer protection against diseases or conditions associated with oxidative damage, such as cardiovascular disease, neurodegenerative disorders, inflammation, aging and cancer (Aggarwal and Shishodia, 2005; book).

Research interest in resveratrol as a chemopreventive and therapeutic agent stems from a landmark report published in the journal *Science* in 1997. In the quest to identify natural plant constituents that may have anti-cancer properties, John Pezzuto's research team at the University of Illinois at Chicago isolated a compound from the Peruvian legume *Cassia quinquangulata* that had apparent activity against cellular events associated with the initiation, promotion and progression stages of carcinogenesis (Jang et al, 1997). The compound, identified as resveratrol, was reported to be the most promising natural, anti-cancer agent of the hundreds that were screened by the Illinois center. Interest in resveratrol in the scientific community and the general public has exploded since the revelations of this initial report. As a measure of the research effort that have been dedicated to further exploring the bioactivity of resveratrol, the number of Pubmed citations for 'resveratrol' have climbed from a total of 59 in 1997 to over 1,600 as of October 2006.

In recent years, studies have reported that resveratrol may be active against various site-specific cancer, including those of the skin, prostate, breast, lung, liver, intestine and colon

(Aziz et al, 2003; review). Numerous biological activities have been ascribed to resveratrol, which may explain its chemopreventive properties. Resveratrol, in addition to possessing anti-oxidant properties, has been shown to inhibit cellular signaling pathways associated with cell survival, block the progression of the cell cycle at various points and induce programmed cell death (apoptosis) (Dong, 2002; review; Perviaz, 2004; review; Ulrich et al, 2005; review).

Although resveratrol has been identified in over 70 plant genera, few of these species are consumed by humans (Pervaiz, 2003). In fact, the richest identified dietary sources of resveratrol remain grapes; grape-associated products, such as red wine and grape juice; and peanuts (Burns et al, 2002). With the exception of red wine, resveratrol is found predominantly conjugated to a glucose moiety (trans- or cis-resveratrol-glucoside) in a structure known generically as piceid (Gu et al, 1999; Vitrac et al, 2002; Rodriguez-Delgado et al, 2002; Gao et al, 2002). Only in rare cases does the concentration of free, resveratrol-aglycone exceed 15 mg/ml in wine. Piceid concentrations can reach levels of 50 mg/ml in wines, but typically are much lower than this. In grapes and peanuts, total resveratrol concentrations rarely exceed 10 µg/g fresh weight and 2 µg/g fresh weight, respectively, with most of this being in the form of piceid (Burns et al, 2002).

There is growing interest in genetically-modifying crops to produce resveratrol for agronomical purposes. A number of crops, including apple (Sparvoli et al, 1994), (Ruhman et al, 2006), rice (Stark-Lorensen, et al, 1997), wheat and barley (Leckland et al, 1998), tomato (Thomzik, 1997), kiwi (Kubayash et al, 2000), alfalfa (Hipskand and Paiva, 2000) and rapeseed (Husken et al, 2005) have been genetically modified to express *resveratrol-synthase*. Most of these crops have expressed resistance against a variety of plant pathogens

and, in addition to offering producer-oriented benefits, through increased crops yields, could offer a variety of health benefits to final users. Thus, metabolic-engineering strategies could be explored as a means to increase the availability of resveratrol-rich foods.

As with natural resveratrol-rich sources, crops genetically modified to express *resveratrol-synthase* accumulate resveratrol, almost exclusively, in the form of piceid (resveratrol-glucoside). Little is known about the bioactivity or bioavailability of piceid, since most of the resveratrol research has focused on the parent aglycone compound due to the commercial unavailability of the glucoside. The limited data available suggests that piceid may be bioavailable (Lv et al, 2006), and as with other phenolic-glucosides, may be a substrate for lactase-phorizin hydrolase, an extracellular enzyme in the small intestines that posseses glucosidase activity (Henry-Vitrac et al, 2006). However, other studies indicate that piceid may not be as biologically available or active as resveratrol-aglycone (Henry et al, 2005); (Kimura and Okuda, 2000).

The studies outlined in this thesis examined the potential chemopreventive activity of a transgenic alfalfa that accumulates piceid (Hipskand and Paiva, 2000) at the site of the colon, using the azoxymethane-induced aberrant crypt foci model of carcinogenesis in CF-1 mice. Aberrant crypt foci are colonic lesions that are widely considered to be preneoplastic, since they are induced by a variety of known colon carcinogens and are highly correlated to risk of colon cancer development in humans (Corpet and Tache, 2002). Previous studies have demonstrated that *trans*-resveratrol-aglycone inhibits the number and size of AOM (Tessitore et al, 1997) and 1,-2-dimethylhydrazine (DMH)-induced ACF as well as the number of DMH-induced tumors in rodents (Sengottuvelan et al, 2006a; Sengottuvelan et al, 2006b; Sengottuvelan et al, 2006c).

Studies assessing the health benefits of genetically modified crops are rare in the literature. A recent example involved the assessment of the influence of a transgenic tomato that accumulates high concentrations of flavonols on cardiovascular health (Rein et al, 2006). In this study, the transgenic tomato fruit peel was shown to significantly reduce basal plasma human c-reactive protein levels in human c-reactive protein transgenic mice relative to the non-transgenic tomato peel. These results demonstrate that the transgenic tomato could provide a strategy to combat cardiovascular disease by providing a rich dietary source of flavonoids.

Genetically modified crops could provide the vehicle to deliver nutraceuticals, such as resveratrol, to a broad population at low costs. In regards to cancer prevention, the administration of a sustainable level of a protective agent over an extended time to a susceptible population via the diet may be a practical and effective intervention strategy, because the onset and progression of cancer occur in multiple stages over many years. Studies in rodent models can provide the initial data to verify the efficacy, in addition to safety, of a GM crop prior to its further development and eventual incorporation into the human diet.

Dissertation Organization

This dissertation examines the efficacy of transgenic, piceid-accumulating alfalfa against colon cancer in an azoxymethane-induced aberrant crypt foci (ACF) rodent model of carcinogenesis. The initial studies with the alfalfa suggested that piceid from the transgenic alfalfa may not be bioavailable. The remaining studies of this dissertation test the hypothesis that piceid is not available, and the addition of β-glucosidase to transgenic-alfalfa as well as

the subsequent liberation of resveratrol-aglycone from piceid is necessary to induce the protective activity of this genetically modified crop in the colon.

The first paper entitled "Transgenic alfalfa that accumulates piceid (trans-resveratrol-3-*O*-β-D-glucopyranoside) requires the presence of β-glucosidase to inhibit the formation of Aberrant Crypt Foci in the colon of CF-1 mice" tests the hypothesis that the transgenic alfalfa would be effective in inhibiting the formation of ACF lesions in CF-1 mice. However, these studies reveal that the supplementation of transgenic alfalfa with exogenous β-glucosidase was necessary in order to achieve a significant reduction in ACF number, indicating that piceid may not be bioavailable. Angela Au, a former Research Associate in Dr. Diane Birt's lab, contributed most of the data for this paper. The data presented in Table 2 of Chapter 2 was conducted in three replicates. Two of these replicates (67% of the mice) were completed by Angela; I was responsible for the third replicate (32% of the mice). For the follow-up studies, comparing the effect of the β-glucosidase supplementation, Angela conducted the first replicate (labeled as Study 1 on Table 3 of Chapter 2). For Angela's studies, Dr. Nancy Paiva at the Samuel Roberts Noble Foundation in Oklahoma was responsible for the transformation and analysis of the alfalfa. For my studies, cuttings from the plants were shipped to Iowa where they were grown outdoors. Dr. Charlie Brummer, a former professor in the Agronomy Deparment at Iowa State University who is now at the University of Georgia in Athens, was involved in helping us grow the plants. I analyzed these plants for piceid accumulation to verify that we were getting values similar to what Dr. Paiva reported in her analysis. I gathered the data, conducted 100% of the data analysis, and wrote the manuscript for this collective work. This paper is currently undergoing a final review for publication in the journal *Nutrition and Cancer.*

The second paper, "Importance of hydrolysis of piceid to resveratrol-aglycone for inhibition of azoxymethane-induced aberrant crypt foci in CF-1 mice fed piceid-accumulating transgenic alfalfa", tests the hypothesis that the added β-glucosidase activity liberated the more bioavailable resveratrol-aglycone from piceid. This study demonstrates that piceid is deglycosylated to a small extent in the gastrointestinal tract and may be a substrate for lactase in the small intestine, as evidenced by the identification of resveratrol-aglycone in the colon of mice fed transgenic alfalfa and transport studies conducted in an *ex-vivo* jejunal system using a lactase-specific inhibitor. However, the colon bioavailabilty of resveratrol-aglycone appears greater than piceid. The supplementation of transgenic alfalfa diets with β-glucosidase appeared to augment resveratrol-glucoside colon bioavailability to levels achieved in mice fed diets with authentic resveratrol-aglycone as illustrated by the similar colon concentrations achieved in both of these groups. Moreover, increased colon bioavailability of resveratrol-aglycone coincided with significant reductions in colonic ACF lesions in these treatment groups compared to relevant controls. For this chapter, I was responsible for 100% of the data collection, data analysis and writing the manscript. Furthermore, I fully developed the concept and experimental approach for the *ex-vivo* jejunal studies. The plants featured in this study were grown in a greenhouse at Iowa State University under Dr. Brummer's supervision. However, I was responsible for the plant crosses, propogation, harvesting and analysis.

The research papers are preceded by a general introduction and a literature review and followed by a general conclusion. The studies cited in the literature review and general conclusion are listed in alphabetical order according to the author's name at the end of the chapter.

Literature Review

Colon Cancer

Colorectal cancer is the term used for any cancer that originates in the colon or rectum of the gastrointestinal tract (American Cancer Society, 2006). Over 95% of colorectal cancers are adenocarcinomas; other colorectal cancers such as lymphomas and squamous cell carcinomas are not as common and not as well characterized (Cuffy et al, 2006). Therefore the research discussed in this review will focus only on studies examining cancers that originate in the colorectal epithelium. It is debatable whether colon and rectal cancers should be considered as a single entity. Although both cancers share many attributes, there is evidence that the etiologies of the two may differ somewhat (Frattini et al, 2004). When possible, this review primarily focuses on cancers originating from the colon.

Epidemiology of Colon Cancer

Colon cancer is one of the most common human malignancies in economically developed societies worldwide as it is the second leading cause of cancer-related deaths in developed countries and the leading cause of cancer-related deaths when smoking-related cancers are excluded (Parkin et al, 2005). The highest incidence rates of colon cancer, based on 2002 data, are in North America, Western Europe and Japan, whereas the lowest rates occur in Africa, Asia and Central America (Parkin et al, 2005). The incidence of and mortality rate for colon and rectal cancer are geographically similar although country differences between rates are more pronounced with colon than rectal cancer.

The incidence and mortality rate of colorectal cancer has decreased gradually throughout the last decade in the United States, but colorectal cancer still remains the third most

common cancer found in men and women living in the U.S. (American Cancer Society, 2006). The American Cancer Society estimates that about 106,680 new cases of colon cancer and 41,930 cases of rectal cancer will be diagnosed in the United States in 2006 (American Cancer Society, 2006). Colon and rectal cancer will account for approximately 55,000 deaths in 2006, affecting males and females equally, based on American Cancer Society estimates.

Laboratory rodent models of colon cancer

Epidemiological studies may provide the empirical justification to further investigate the anti-cancer properties of a dietary constituent. Since it is frequently not ethical or practical, due to time limitations and costs, to assess the potency and mechanism of the action of potential chemopreventive or therapeutic agents in humans, initial studies are often performed in rodent models of colon carcinogenesis.

Spontaneous epithelial tumors of the colon are very rare in experimental animals (Rangaajan and Weinberg, 2003). To induce colon tumors, laboratory rodent strains are injected with carcinogens, which most commonly consists of dimethylhydrazine (DMH) derivatives. Dimethylhydrazine is metabolized to azoxymethane (AOM) and methylazoxymethane in rodents (Macejova and Brtko, 2001). AOM is injected directly into the circulation of rodents and is usually preferred over DMH because it is more potent and is more stable in chemical solutions (Papanikolaou et al, 1995). Endoscopically visible colon tumors can be detected as early as 15 weeks in rats injected with AOM (Reddy, 2004). AOM treatment induces tumors predominantly in the distal region of the colon, and roughly 70% of these tumors are adenocarcinomas (Reddy, 2004).

Since tumor studies are very costly and time consuming, shorter-term experiments are usually carried out using purported precancerous colonic lesions as biomarkers to screen dietary constituents or synthetic compounds for any pro- or anti- colon cancer activity. Currently, the most utilized and accepted pre-cancer markers for the colon are aberrant crypt foci (ACF). ACF are abnormally large crypts that are relatively easy to identify under a light microscope (Bird, 1995). They are defined as being elevated crypts that are at least two to three times larger than normal crypts and have a thick epithelial lining that can be stained darker than normal crypts with methylene blue. ACFs are widely regarded as preneoplastic lesions because they are induced by all colon carcinogens, including AOM, in a dose-dependant manner. They also predict tumor outcome in several rodent studies and correlate with cancer risk in humans (Corpet and Tache, 2002). Furthermore, ACFs in humans have a morphology similar to those examined in rodents.

Some studies have reported conflicting results between ACF number and tumor development. For example, hundreds of lesions are induced by AOM in rats, yet the number of tumors that eventually form are relatively few (Mori et al, 2004). Additionally, some chemopreventive agents, such as genistein and 2-(carboxyphenyl) retinamide, effectively suppress ACF induction by AOM, but do not suppress and may even facilitate the development of colon cancer (Zheng et al, 1999; Gee et al, 2000). Based on these observations, it has also been proposed that ACF are heterogeneous in nature, with two types occurring: non-dysplastic and dysplastic ACF (Mori et al, 2004). It is estimated that roughly 5% of AOM-induced ACF in rats are dysplastic (Jen et al, 1994). The associated mutations for non-dysplastic and dysplastic lesions in rats also appear to be different. Most non-dysplastic ACF express a *k-ras* mutation, whereas dysplastic lesions harbor

adenomatous polyposis coli (APC) mutations (Takahashi and Wakebayeshi, 2004). However, this characteristic is not observed in mice, where k-ras mutations in AOM-treated animals appear rarely. As of the time of this writing, ACF in mice largely remain uncharacterized.

In recent years, additional biomarkers have been proposed to be possible dysplastic subsets of ACF (Mori et al, 2004). Abnormal activation of the APC/β-catenin/TCF/Lef pathway (Wnt pathway) is documented to be associated with decreased membrane levels of β-catenin accompanied by the accumulation of β-catenin in the cytoplasm at the cellular level (Logan et al, 2004). The accumulation of the β-catenin protein can be observed in certain ACF following histological examination. The resulting lesions have been coined "β-catenin accumulating crypts (BCAC)" and may better represent preneoplasic lesions (Yamada, 2003). However, the tissue preparation necessary to score colons for such lesions is tedious, and it is not practical to view entire colons for these markers. In addition to BCAC, mucin-depleted foci (MDF) are purported preneoplastic lesions that may correlate better with tumor development than ACF (Mori et al, 2004). MDF are characterized by absent or scarce mucous production than can be easily identified microscopically along the entire mucosal surface of colons by tissues stained with high-iron diamine alcian blue. Alcian blue is a cationic dye that reversibly binds to the negatively-charged carbohydrate mucin. Whole colon tissues can be stained with methylene blue and scored for ACF number and also de-stained with methanol and then re-stained with alcian blue to identify ACF that are MDF. Unlike ACF, the number of MDF in AOM-treated rats are very low and are in the magnitude of tumors induced by the same dosage of the carcinogen. Additionally, MDF are more likely to exhibit over-expression of β-catenin than ACF (Femia et al, 2005). In fact,

MDF comprising more than 4 crypts may well correspond to BCAC. Currently, however, ACF are still widely accepted as the "gold standard", short-term biomarker of colon cancer as BCAC and MDF have been featured in very few studies.

In addition to the carcinogen-induced colon lesions discussed above, transgenic rodent models have been identified which spontaneously develop gastrointestinal adenomas and have been used in studies examining the efficacy of potential chemopreventive agents (Green and Hudsen, 2005). In 1990, a mutant mouse, Min, was found to have multiple intestinal neoplasia caused by a truncated APC protein (Moser et al, 1990). These mice have since been used in a number of rodent cancer studies. However, the major drawback of this model is that mice that are heterozygous for the mutated APC gene develop tumors in the small intestine, not in the colon; mice that are homozygous for the mutant APC gene die *in-utero* (Green and Hudsen, 2005). Moreover, most dietary treatment regimes followed in studies using these mice begin with mice that are 4-5 weeks old when tumors are already present, which represents a more advanced stage of cancer (Corpet and Tache, 2002). Neoplastic lesions are observed *in utero* in Min mice.

Etiology of Human Colon Cancer

Genetic factors

Cancer is a disease that initiates from the acquisition of mutations at the cellular level with the sum of the mutations resulting in cells that divide and invade surrounding tissues and organs. In colon cancer, mutations are known to occur via inherited germline mutations or are acquired secondarily following an environmental insult. About 20-25% of colorectal cancers occur in familial (hereditary) patterns (de la Chappelle, 2004). The two best known and characterized conditions of nonsporadic colon cancer are Familial Adenomatous

Polyposis (FAP) and Hereditary Non-Polyposis Colorectal Cancer (HNPCC), also known as Lynch Syndrome. A germline mutation in the adenamatous polyposis coli (APC) tumor-suppressor gene is the likely cause of APC, which is characterized by the development of thousands of adenomas throughout the colorectal region of an individual, beginning in late childhood or early adolescence. If left untreated, at least one adenoma will develop into an adenocarcinoma. Germline mutations in the mismatch repair genes MCH1, MSH2, MSH6 and PNS2 are the purported cause of HNPCC. Unlike APC, HNPCC is not limited to colorectal cancer. In addition to colorectal cancer, individuals with HNPCC are predisposed to developing cancers of the endometrium, stomach, ovaries, small bowel, hepatobilliary epithelium, uroepithelium and the brain. The genetic syndromes APC and HNPCC, collectively, account for about 5% of all colon cancer cases.

Sporadic colorectal cancers account for the vast number of cases diagnosed each year (around 75%) (Hendon and DiPalma, 2005). Based on epidemiological data, approximately 5 to 7 oncogene mutations are necessary before the cellular transformation of a normal cell to a cancerous cell occurs (Kinzler et al, 1996; Calabrese et al, 2005). If one or more of these mutations are present at birth as a germline mutation, fewer mutations are required for complete transformation. Vogelstein and colleagues have proposed a multi-step model based on clinical data for the specific genetic events that potentially account for the progression of colorectal colon cancers (Fearon and Vogelstein, 1990). This model offers an explanation of the observed normal mucosa to adenoma to carcinoma sequence of morphological events that can be observed in biopsies from patients at various stages of colorectal cancer. According to the Vogelstein model, a mutation in the APC gene initiates the neoplastic process. Tumor progression results from the mutation of other genes prior and after this

initial event, since a mutation of APC alone does not seem to lead to dysplastic growth. A mutation in k-Ras is likely to occur early, following mutations in APC. Ras is a component of the MAP kinase single transduction pathway and is involved in regulating the rate of cellular division. Tumors in 20% of all human cancers and 50% of colon cancers harbor ras mutations (Halaschek-Wiener et al, 2004). Mutations in genes associated with "housekeeping" or tumor suppressor roles, such as the mismatch repair genes and p53, seem to occur at later stages. The tumor suppressor p53 appears to function DNA repair by halting the progression of the cell cycle to allow various DNA repair enzymes a chance to fix any mutated sequences in DNA (Johnson and Walker, 1999). Overall, p53 is mutated in about half of all human cancers (Midgley and Kerr, 1999).

Diet and Lifestyle factors

In a 1981 report, Doll and Peto estimated that 75-80% of all cancer deaths in the United States could be avoided by dietary modifications (Doll and Peto, 1981). Findings from migrant studies of populations moving from areas of low incidence of colorectal cancer to areas of high incidence reveal that environmental factors play a major role in the development of colorectal cancer. For instance, the incidence rate of colorectal cancer in U.S.-born Japanese men has been reported to be twice that of foreign-born Japanese men and 60% higher than Caucasian U.S.-born men (Flood et al, 2000). This same trend has been shown with Korean, Filipino and Chinese populations that immigrated to the U.S. (Gomez et al, 2003).

As with other site specific cancers, various dietary constituents and lifestyle behaviors have been implicated as being possible determinates in the risk of developing colon cancer. Several observational studies suggest that diets low in energy and high in certain

micronutrients such as vitamin D and selenium may be protective against colon neoplasia, while high red meat intake (especially well-done meat), high alcohol consumption, physical inactivity and cigarette smoking increasing the risk of colorectal cancer (Potter, 1996; Lipkin et al, 1999). Certain subtypes of fat may be related to risk, as high intake of red meat has been linked with colon cancer risk in some studies, whereas no correlation has been observed between the consumption of other foods rich in fat, such as poultry, fish and dairy products (reviewed by Giovannucci and Goldin, 1997). However, it is very difficult to control for other dietary constituents that may skew results when examining the health benefits or adverse effects of dietary fat subtypes.

Fruits and vegetables have traditionally been thought to be protective against colon cancer. Case-control studies have largely supported a protective role of vegetables and fruits against colon cancer (World Cancer Research Fund, 1997). However, higher intakes of fruits and vegetables (more than 5 servings per day) were not associated with lower risk of colon cancer in a prospective study featuring 62,602 men and 70,554 females (McCullough et al, 2003). Individuals who consumed less than 1.3 servings of fruits and vegetables per day where at a higher risk of developing colon cancer relative to individuals who consumed more than 5 servings of fruits and vegetables per day ($p=0.06$). In contrast to this study, the Nurses Health Prospective study, which included 71, 910 female and 37,725 male subjects, did not reveal a causal relationship between decreased fruit and vegetable intake and increased risk of cancer even though an inverse trend between fruit and vegetable consumption and risk of cardiovascular disease was observed (Hung et al, 2004).

Epidemiological data does suggest that increased intake of folate, a nutrient rich in plant-based foods, may be protective against colon cancer, but intervention studies in humans are

inconclusive on this matter (Kim, 2003). Vitamins C and E and ß-carotene, which are also abundant in plants, have purported anti-oxidant activity, but case-control studies in humans do not support a protective role of any of these constituents against colon cancer (Greensberg et al, 1994). The link between colon cancer and dietary fiber, another component of fruits and vegetables that may contribute to protective activity, is controversial as most large prospective studies have indicated no protective effect. A 16 year follow-up of 88,757 women did not reveal an association between intake of dietary fiber and the risk of colon cancer, since the relative risk of colon cancer was 0.95 (95% confidence interval; 0.73 to 1.25) in individuals who consumed less than 10 g of fiber per day (Fuchs et al, 1999). Data analysis from the Alpha-Tochopherol Beta-carotene Prevention study of 27, 111 male smokers did not show a significant difference in relative risk of colorectal cancer between individuals who consumed more than 34 g of fiber per day verses subjects that consumed less than 16 g per day (Pietine et al, 1999). An inverse association between consuming few fruits and vegetables (less than 1.5 servings per day) and risk of colorectal cancer was reported in a 10 year follow-up study of 61, 463 individuals, but no association was found with subjects who consumed less than 6 g of fiber per day (Terry et al, 2001). In contrast to these studies, the large European Prospective Investigation of Cancer and Nutrition Study that examined a heterogeneous population from ten countries showed a reduction in colon cancer risk between groups in the highest fiber quintile (35 g per day) vs. the lowest quintile (15 g per day), after adjusting for folate (Bingham, 2006). Animal studies and case control experiments in humans suggest a protective role of soluble fiber, but do not support a protective role for insoluble fibers against colon cancer (reviewed by Pool-Zobel, 2005).

Vegetables and fruits are also rich in non-nutrient bioactive compounds called phytochemicals (Acamovic et al, 2005). Phytochemicals encompass diverse classes of compounds, including flavonoids, flavones, phenols and polyphenols. These compounds may be produced in plants for the purpose of attracting or repelling harmful organisms and providing protection against UV light and abiotic environmental stresses in addition to other unknown functions (Liu, 2004).

A few plant polyphenolic compounds have been isolated and have been shown to possess potent antioxidant and potential chemopreventive and therapeutic activities (Waladhani et al, 1998, review; Sun et al, 2002, review; Chu et al, 2002, review). Compounds, including phenolic acids, flavonoids, catechins, anthocyanins and stilbenes, have been reported to induce cell differentiation and apoptosis, inhibit cell proliferation by regulating genes involved in this process or by initiating cell cycle arrest, modulate the activities of enzymes involved in the detoxification of xenobiotics,regulate hormonal fluxes and elicit anti-bacterial or anti-viral activity (Waladhani et al, 1998, review; Sun et al, 2002, review; Chu et al, 2002, review).

Dietary sources of potential health-benefiting phytochemicals vary greatly depending on the class of compounds. For example, flavonols such as quercetin and anthocyanins, which provide the pigmentation of fruits and flowers, are found in a wide variety of edible plant species (Manach et al, 2004). On the other hand, the stilbene compounds are found in very few food sources and in very low concentrations (Scalbert and Williamson, 2000). The concentration of polyphenolic phytochemicals can vary within a particular plant species due to ripeness, time of harvest and environmental factors such as processing and storage (Manach et al, 2004). Most epidemiological studies do not differentiate daily intake of fruits

and vegetables based on storage or processing conditions (i.e., fresh vs. frozen) or in what manner foods were processed (Scalbert and Williamson, 2000). Frozen vegetables may have lower phenolic concentrations and antioxidant capacity relative to fresh vegetables (Ninfali and Bacchiocca, 2003) and, since phytochemicals are not evenly distributed throughout a plant, food fractionation that can occur during processing can result in the loss or enhancement of phytochemicals (Burda et al, 1990). Furthermore, very little is currently known about the phytochemical composition of specific foods (Brat et al, 2006). It is possible that the inconsistent findings between prospective and case-control studies in regards to the potential anti-cancer activity of fruits and vegetables in the colon could be the result of the limitations in estimating daily intake of phytochemicals.

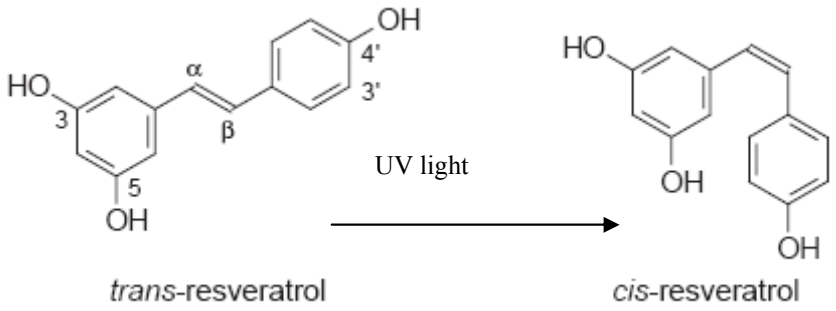
Resveratrol

One of the most studied polyphenolic compounds in recent years is resveratrol – a phytochemical that is produced by a few plant species in response to injury and external stresses such as UV light and fungal infection and is found in some natural and processed food and beverage products such as grapes, peanuts, red wine and grape juice (Fremont, 2000). Research interest in resveratrol stems from epidemiological studies showing an inverse correlation between red wine consumption and incidence of cardiovascular disease (Renaud and de Lorgeril, 1992). Since resveratrol was identified as a consistent constituent in red wine, numerous *in vitro, in vivo* and *ex vivo* experiments have been carried out and have demonstrated that this compound can trigger a wide array of biological activities. In addition to providing possible protection against cardiovascular disease, resveratrol may also have cancer chemopreventive and therapeutic benefits, and may protect against other

chronic diseases or conditions associated with oxidative damage, such as neurodegenerative disorders and aging (Aggarwal and Shishodia, 2005; book).

Chemical Structure (s)

Resveratrol is a member of the stilbene class of polyphenolic compounds produced as secondary metabolites by plant species (Jeandet et al, 2002). All stilbenes have as their base structure two benzene rings connected by a carbon-carbon double-bond bridge. Resveratrol is the common term for 3,5,4'-trihydroxystilbene. Hence, resveratrol is a stilbene with three-hydroxyl moieties attached at the 3,5 and 4' carbons on the stilbene- base structure. Resveratrol is an off-white powder with a melting point of 253 to 255°C, a molecular formula of $C_{14}H_{12}O_3$ and a molecular weight of 228.25 g/mol (Fremont, 2000). Resveratrol exists in both trans and cis isomeric forms (Figure 1). Isomerization to the cis form is known to be induced by exposing the *trans* form of resveratrol to UV light (Fremont, 2000). The ultraviolet (UV) absorption maximum (λ_{max}) for the *trans* and *cis* isomers are 308 and 288 nm, respectively, which allows for their detection and separation via high-performance liquid chromatography (HPLC). Since *trans*-3,5,4' hydroxystilbene is the most extensively studied form of resveratrol, throughout this review the term "resveratrol" will be applied to refer specifically to the *trans*-3,5,4' hydroxystilbene isomer.



In plants, resveratrol is found predominantly as a 3′-*O*-*β*-D-glucoside, also commonly known as piceid (Fremont, 2000). Other conjugated forms of resveratrol have been isolated from plants, including a 4′-*O*-*β*-D-glucoside and resveratrol with 1-2 methyl groups, a sulfate or a fatty acid (Pervaiz, 2003). However, these are very minor forms of resveratrol. It is believed that the addition of the glucose moiety to resveratrol protects it from enzymatic oxidation in nature, and thus extends the half-life and antioxidant capabilities of the compound in the plant cell (Regev-Shoshanini et al, 2003).

Natural sources

To date, resveratrol has been identified in a limited number of plants (about 70 genera) (Pervaiz, 2003). Resveratrol was first identified in 1940 as a constituent of the roots of white hellebore (*Varatrum* grandiflorum) and later, in 1963, as an active constituent of the Japanese and Chinese folk medicine Ko-jo-kon, which is the dried powdered root of Japanese knotweed *Polygonum cuspidatum* (cited in Seeram et al, 2006). Resveratrol has also been documented in eucalyptus and spruce trees and has been identified in a few flowering plants, such as *Veratrum grandiflorum* and *Veratrum formosanum* (Pervaiz et al, 2003).

Several methods have been used to extract resveratrol and related compounds from plant, foods and beverages. They include high-performance liquid chromatography (HPLC) (Goldberg et al, 1997), gas chromatography (Lin and Chen, 2001), gas chromatography-mass spectrometry (Soleas et al, 2001) and capillary electrophoresis (Lin and Chin, 2001). As extraction methods and detection limits have improved, resveratrol and piceid have been identified in various foods and beverages (Table 1).

The first report of resveratrol detection in grapevines (*Vitis vinifera*) occurred in 1976 (Langcake and Pryce, 1976).

Table 1: Dietary and natural sources of resveratrol (from Bauer and Sinclair, 2006, with some modfications)

	Trans-resveratrol concentration	Comments
Red wines	0.1-14.3 mg l^{-1}	*Cis*-resveratrol, *trans*- and *cis*-piceid also present; usually at lower concentrations
White wines	<0.1-2.1 mg l^{-1}	*Cis*-resveratrol, *trans*- and *cis*-piceid also present; usually at lower concentrations
Grapes	0.16-3.54 ug g^{-1}	Contents are similar for wine or table grapes, and black or white grapes, *trans*-picied predominant at concentrations of 1.5-7.3 ug g^{-1}
Red grape juices	0.50 mg l^{-1}	*Trans*-piceid, *cis*-piceid and *cis*-resveratrol found at concentrations of 3.38 mg l^{-1}, 0.79 mg l^{-1} and 0.06 mg l^{-1}, respectively
Blueberries	Up to 32 ng g^{-1}	
Peanuts	0.02-1.92 µg g^{-1}	
Itadori (*Polygonum cuspidatum*) tea	0.68 mg l^{-1}	*Trans*-piceid found at concentration of 9.1 mg l^{-1}
Polygonum cuspidatum (herb root)	0.524 mg g^{-1}	*Trans*-piceid found at concentrations of 1.65 mg g^{-1}

In the years following this discovery, resveratrol has been intensely studied in grapes. In grapes and berries, resveratrol is mainly found in the skin, not in the flesh (Siemann and Creasy, 1992). As Table 1 shows, the resveratrol content of grapes can vary dramatically.

The concentration of resveratrol in grapes can depend on a variety of factors, including the type of grape, the climate in which the grape was grown and the time of harvest (Careri et al, 2003). Increasing irradiation of harvested grapes by ultraviolet B (UVB) or UVC light is also known to enhance yields of resveratrol (Cantos et al, 2000). Other berries, such as blueberries, cranberries and billberies, contain resveratrol, but only at trace amounts (less than 30 ng g^{-1}) (Lyons et al, 2003). Since the maceration process of red wine production is significantly longer than that of white wine, red wines consistently have much higher concentrations of resveratrol than white wines (Fremont, 2000).

In wine, *cis* and *trans* isomers of resveratrol are present in the free or glycosylated forms at significant but lower concentrations than the aglycone (Gu et al, 1999; Vitrac et al, 2002; Rodriguez-Delgado et al, 2002; Gao et al, 2002). *Cis*-resveratrol has not been reported in grape skins. It is assumed that the formation of the *cis*-isomer in wines occurs by the isomerization of the *trans* form on exposure to light and oxygen. Resveratrol has been predominantly identified in a glycosylated form (piceid) in plants and fruits where different isoforms of resveratrol have been assessed (Burns et al, 2002). *Polygonum cuspidatum* remains one of the richest natural sources of resveratrol, which has been used for centuries to treat a number of ailments, including dermatitis, gonorrhea favus, athlete's foot and hyperlipidemia (Shishodia and Aggarwal, 2006). However, the resveratrol found in *Polygonum cuspidatum* is almost exclusively in the form of piceid (Vastano et al, 2000).

In addition to the natural and processed sources mentioned, a variety of synthetic schemes have been reported for the production of purified resveratrol (Farina et al, 2006). Not surprisingly, various companies now sell resveratrol as a natural, unregulated supplement (Bagchi, 2000).

Biological Activities of Resveratrol

Epidemiological evidence from many studies overwhelmingly supports the notion that light to moderate consumption of red wine is associated with a reduction in mortality due to cardiovascular disease (Renaud and Lorgeril, 1992). This relationship has been popularly coined "The French Paradox" or "The French Connection" because red wine consumption has been associated with the French who have a low incidence of cardiovascular disease although they consume a diet that is relatively high in saturated fat. Red wine is rich in a number of biologically active phytochemicals that could act individually or synergistically to provide cardiovascular benefits (German and Walzem, 2000).

Since its discovery in red wine in 1992 (Siemann and Creasy, 1992), resveratrol has been shown to possess a remarkable range of biological activities that reveal a possible role in protection against cardiovascular disease. Resveratrol has been shown to reduce the copper-catalyzed oxidation of human low-density lipoproteins at a dose of 10 μM in an ex-vivo system (Frankel et al, 1993), induce vasorelaxation in human endothelial cells at a dose of 30 μM (Li et al, 2000) and inhibit platelet aggregation and polymorphonuclear cell activation induced by collegen *in-vitro* in a dose range of 10-1000 μM (Wang et al, 2002; Rotondo et al, 1998). In addition to cardiovascular disease, resveratrol may be protective against other diseases and conditions associated with oxidative damage such as

neurodegenerative disorders (Han et al, 2006), inflammation (Surh, 2003) and cancer (Dong, 2002; review; Perviaz, 2004;review; Ulrich et al, 2005; review).

The first report on the possible bioactivity of resveratrol against chemical-induced carcinogenesis surfaced in 1997. In a landmark paper, Jang et al reported that resveratrol inhibited free-radical formation and incorporation of [^{3}H] thymidine in promyelocytic leukemia (HL-60) cells, induced quinine reductase activity in cultured mouse hepatoma cells and, in a dose-dependant manner, inhibited DMBA-induced tumors in the mammary gland of female CD-1 mice (Jang et al, 1997). Collectively, these findings demonstrated that resveratrol can inhibit cellular events associated with each stage of carcinogenesis – initiation, promotion and progression. This report strongly suggested that resveratrol may be a potent chemopreventive and chemotherapeutic agent. In the years following the studies by Jang et al, many studies in cell culture systems and animal models have verified a chemopreventive or therapeutic role of resveratrol in various tissues including the prostate, breast, liver, digestive tract and lung (Aziz et al, 2003, review).

The activities of other isoforms of resveratrol are rarely discussed in the literature. By most indications, the *cis* isomer of resveratrol seems to be less active than the *trans* isomer. The *trans* isomer appears to inhibit cyclooxygenase (COX)-1 activity, COX-2 activity and the development of 7,12-dimethylbenz[*a*]anthracene-induced preneoplasic lesions in mouse mammary glands more effectively than the *cis* isomer (Pettit et al, 2002). Stivala et al reported that *trans*-resveratrol inhibited the proliferation of normal human fibroblasts and HT1080 cells, whereas *cis*-resveratrol had no significant effects on cell growth at the concentrations tested (Stivala et al, 2001). It was also shown in this study that *trans*-

resveratrol, unlike *cis*-resveratrol, inhibits DNA biosynthesis in fibroblasts and inhibits the activity of B-type DNA polymerases α and δ.

The bioactivity of piceid has been more rarely analyzed, probably because the purified compound was not commercially available until very recently. Using trolox, a water-soluble derivative of Vitamin E, Kerem et al. examined the potential anti-oxidant and α-glucosidase inhibitory activities of *trans*-resveratrol, piceid and a novel *trans*-stilbene (5,4'-dihydroxystilbene-3-*O*-α-arabinopyranoside) (rumexoid), all of which were isolated from the roots of *Rumex bucephalophorus* (Kerem et al, 2006). The antioxidant capacity of resveratrol was slightly higher than that of piceid, 2.7 vs. 2.4, expressed as trolox equivalent antioxidant capacity. Rumexoid had the lowest antioxidant capacity (1.5 trolox equivalents). However, piceid did not significantly inhibit α-glucosidase activity at the concentrations tested (0.05-0.2 mM), unlike resveratrol and rumexoid (IC50 < 0.1 and < 0.5 mM, respectively).

Kimura and Okuda studied the effects of piceid on tumor growth and lung metastasis in Lewis lung carcinoma (LLC)-bearing mice (Kimura and Okuda, 2000). Piceid, which was isolated from the roots of *Polygonum cuspidatum,* did inhibit tumor growth in the right hind paw of mice injected with LLC cells, but at the very high dose of 300 mg (770 μmol)/kg body weight which was administered orally twice daily for 32 days, starting 12 hours after tumor implantation. Piceid inhibited DNA synthesis in LLC cells at a concentration of 1,000 uM, but not at lower concentrations (10 to 100 uM).

Seemingly, the activity of piceid has not been examined against other site-specific cancers, nor has the chemoprotective activity of piceid and resveratrol-aglycone been compared in a side-by-side study, since no other such studies could be located in the

literature when preparing this review. With only a few studies to consider, it is possible that piceid has some chemopreventive properties, but has less activity than *trans*-resveratrol. However, this is purely speculative at this point, because all of the studies examined used very high doses of piceid.

Resveratrol and colon cancer

Two carcinogen-treated animal models, azoxymethane and 1,2-dimethylhydrazine, have been used to assess the antitumor effects of resveratrol in the colon as a model reproducing the development of sporadic colorectal cancer in humans. In addition, Min mice have been used as a model for familial adenomatous polyposis.

Tessitore et al. reported that resveratrol affected the formation of AOM-induced aberrant crypt foci in F344 rats administered with resveratrol at a dose of 200 ug (0.89 μmol)/kg body weight/day in the drinking water with 0.2% ethanol for 100 days beginning 10 days before two AOM injections (15 mg/kg body weight) (Tessitore et al, 2000). In this study, resveratrol significantly reduced the number (25.7 ± 3.6 vs. 39.4 ± 3.3) and multiplicity (number of crypts/focus) (2.7 ± 0.3 vs. 4.9 ± 0.6) of aberrant crypt foci (ACF) in the colonic mucosa of the rats.

Recently, resveratrol was reported to inhibit full-blown colon carcinogenesis (Sengottuvelan et al, 2006a). In this study, resveratrol at a dose of 8 mg (36 μmol)/kg body weight per day, intragastrically, in a 1% (w/v) carboxymethylcellulose/water solution reduced the incidence and size of 1,-2-dimethylhydrazine (DMH)-induced tumors in the colonic mucosa of Wistar rats after a 30-week treatment period. Rats were administered resveratrol either during the initiation (beginning one week before DMH injections until one week after final exposure to DMH), post-initiation (beginning two days after DMH

injections and lasting for the remaining 30 weeks of the study) or the entire phase of the study. Resveratrol was shown to be most effective in inhibiting the number of DMH-induced tumors when it was administered throughout the duration of the study or during the post initiation phase.

Results of resveratrol chemoprevention studies in Min mice have been inconsistent. Resveratrol (12-16 mg (53-71 μmol)/kg body weight) was administered in the drinking water over a seven week period to Min mice (Schnieder, 2001). The formation of intestinal tumors was reduced by 70% compared to mice that received drinking water with 0.4% ethanol, alone (Schnieder, 2001). On the other hand, resveratrol at daily doses lower than 90 mg (400 μmol)/kg body weight had no effect on tumor incidence in the small intestine of Min mice in other studies regardless of whether resveratrol was administered in 10% ethanol in water or via a solid diet vehicle (Ziegler et al, 2004; Sale et al, 2005). However, at the high dose of 240 mg (1 mmol)/kg body weight per diem, resveratrol did reduce the number of intestinal adenomas by 27% in one of these studies. The results of Schneider et al. are perplexing as no feasible explanation can be offered to account for the differences in the final outcome of that study with the results of the studies of Ziegler et al and by Sale et al. All studies conducted in Min mice were initiated in mice that were 5-6 weeks of age, which probably represents a more advanced stage of cancer in this model compared to AOM- and DMH-treated rats. The discrepancy of results obtained with Min mice suggests that resveratrol may be more effective as a chemopreventive agent than as a therapeutic tool.

Chemopreventive mechanisms of resveratrol

Biochemical pathways involved in cell-cycle regulation, apoptosis, angiogenesis and differentiation (Ragione et al, 1998; Della et al, 2002; Bruder et al, 2001; Nielson et al,

2000) have all been identified as being potential targets of resveratrol. The complexity and multiplicity of molecular targets of resveratrol in the intestine was illustrated in the study by Schneider et al, where the expression of 588 genes in the small intestine of Min mice administered with resveratrol was compared to control Min mice (Schneider et al, 2001). In that study, resveratrol was found to down-regulate genes that are directly associated with cell cycle progression or cellular proliferation (cyclins D1 and D2, DP-1 transcription factor and Y-box protein) and up-regulate genes involved in the recruitment and activation of immune cells (cytotoxic T lymphocyte Ag-4, leukemia inhibitory factor receptor and monocyte chemotactic protein 3) and in the inhibition of tumor progression. It is currently not known if the apparent chemopreventive properties of resveratrol are the result of its ability to affect the expression of a number of proteins in the cell or the result of resveratrol's ability to influence specific pathways important in carcinogenesis.

The ability of resveratrol to prevent the initiation of carcinogenesis may, at least partially, be attributed to its antioxidant activity. DNA can undergo attack by different reactive oxygen species (ROS) generated during metabolic processes or physical activation of oxygen by irradiation (e.g. UVB in sunlight). Chemicals with antioxidant activities may prevent or delay the onset of cancer by blocking ROS generation. Resveratrol (75 μM) inhibited lipid peroxidation in RAW 264.7 macrophages exposed to $OH^{\bullet}$ radicals (Leonard et al, 2003) and, at a dose of 25 μg/ml (109 μM), induced an increase in glutathione reductase levels in a concentration-dependent manner in human lymphocytes activated H_2O_2 (Olas et al, 2004).

Resveratrol may also block the initiation of carcinogenesis through the inhibition of cyctochrome P450 monooxygenases CYP1A1 and CYP1B1 (Piver et al, 2003), which

catalyze the oxidation of xenobiotics. Reactive metabolites, which can form DNA adducts and cause mutations, are formed as by-products of these cytochrome P450 catalyzed reactions. Resveratrol down-regulates the expression of these enzymes by inhibiting the binding of the arylhydrocarbon receptor (AhR) to xenobiotic response elements (XRE) on DNA (Ciolino et al, 1998). On the other hand, resveratrol has been shown to induce expression of Phase II enzymes such as heme oxygenase I and quinone reductase, which are capable of detoxifying carcinogens (Heo et al, 2001).

In recent studies, Sengottuvelan et al. demonstrated that resveratrol at a daily dose of 8 mg/kg body weight in the drinking water inhibited DMH-induced ACF and tumors after 30 weeks through a process that may involve the induction of anti-oxidant enzymes, such as superoxide dismutase and catalase, in the colorectal mucosa (Sengottuvelan et al, 2006b) and reducing activity of enzymes involved in biotransformation in the colonic flora and mucosa (Sengottuvelan and Nalini, 2006c). In the later study, the fecal activity of β-glucuroniase, β-glucosidase and β-galactosidase were elevated in control-fed, DMH-injected rats. Resveratrol reduced the fecal and mucosal activities of these enzymes to levels observed in control rats that were not injected with DMH. These enzymes may catalyze the hydrolysis of conjugated carcinogens, releasing the toxic versions of these compounds. In addition to this, resveratrol reduced the mucinase of the colonic flora. Mucinase hydrolyzes mucin, which provides a protective coating lining the intestinal luman wall, exposing the mucosa to potential carcinogens. Gut flora microflora β-glucosidase and mucinase activity may be important pre-cancer events of the colon (Hambly et al, 1997).

Resveratrol has been shown to suppress proliferation in a number of immortalized cell lines (Aziz et al, review). Resveratrol, when administered to Wistar rats at a daily dose of 8

mg (36 μmol)/kg body weight for 30 weeks, significantly reduced the number of argyrophilic nucleolar organizing region-associated proteins per nucleus in non-lesional colonic crypts by 30%, which suggests that resveratrol, at least in part, reduced the number of 1,-2-dimethylhydrazine (DMH)-tumors by reducing cellular proliferation (Sengottuvelan et al, 2005). The anti-proliferative activity of resveratrol could be attributed to its ability to reduce the activities of ribonucleotide reductase (Fontecave et al, 1998) and DNA polymerase activity (Locatelli et al, 2005). Resveratrol can also inhibit cellular proliferation by inducing cell cycle arrest. A number of studies have demonstrated that resveratrol induces cell cycle arrest in the G1/S phase in breast, prostate, colon, esophageal and leukemia cell lines (Hsieh et al, 1999 a and b; Joe et al, 2002; Kuwajerwala et al, 2002; Park et al, 2001). However, a G1/M phase arrest in HepG2 liver cancer cells (Kuo et al, 2002) and G2/M phase arrest in SW480 human colon cells and WR-21 human salivary adenocarcinoma cells (Delmas et al, 2002; Young et al, 2006) have been reported. Some studies have also found that resveratrol-induced cell cycle arrest is reversible (Ragione et al, 1998; Haider et al, 2003; Della et al, 2002).

Resveratrol also induced S-phase cell cycle arrest in Caco-2 human colon carcinoma cells treated with a dose of 25 μM for 16 and 24 hours in one study (Schneider et al, 2000) and at doses higher than 50 μM after 24 hours treatment in another study. In SW480 human colon cancer cell lines, resveratrol induced S-phase cell cycle arrest at a dose of 30 μM after 24 hours treatment and at a lower dose of 10 μM following 48 and 72 hour treatments (Delmas et al, 2002). The mechanism behind resveratrol's ability to inhibit cell cycle progression at the S phase seems to be due to the up-regulation of p53, an oncosuppressor protein that activates the cyclin inhibitor p21 (Hsieh et al, 1999; Kim et al, 2003; Kim et al,

2004). Generally, up-regulation of p53 in resveratrol-treated cells is also accompanied by the accumulation of cyclins E and A, decreased levels of D-type cyclins and dephosphorylation and thus the activation of the tumor suppressor retinoblastoma protein (pRb) (Wolter et al, 2001; Adhami et al, 2001; Kim et al, 2003). In Caco-2 and SW480 cells, resveratrol induced the expression of cyclins A, B and E and reduced the expression of cyclin D. However, resveratrol at doses higher than 50 uM reversed S-phase arrest in Caco-2 cells, possibly through a mechanism that involved reversed changes in the phosphorylation status of the retinoblastoma protein (Wolter et al, 2001).

Resveratrol may also induce apoptosis in cancerous cells by modulating the expression of proteins important in the apoptotic process. Mitochondrial-mediated apoptosis is facilitated by the proapoptitic Bcl-2 family proteins, such as Bax and Bid. Tessitore et al. reported that in resveratrol-treated rats, Bax expression was enhanced in the colonic mucosa of aberrant crypt foci, but not in the surrounding mucosa (Tessitore et al, 2000). It was further demonstrated in this study that p21 expression was lost in the normal mucosa of resveratrol-treated rats, but was expressed in the normal mucosa of controls and in the ACF of both groups. However, resveratrol has also shown to induce apoptosis in both HCT116 cells with knockout Bax and in HCT 116 cells that expressed wild-type Bax (Mahyar-Roemer et al, 2002). In HCT116 Bax -/- cells, apoptosis was reduced but not absent, which suggests that resveratrol can induce a Bax-mediated and a Bax-independent mitochondrial apoptosis in the colon.

Another mechanism whereby resveratrol may provide protective activity against carcinogensis could be through its ability to prevent the synthesis and/or activity of enzymes that are considered carcinogenic when over-expressed. Cyclooxygenases 1 and 2 catalyze

the first committed step of prostaglandin H_2 from arachidonic acid. Prostaglandin H_2, in turn, is converted to prostaglandin E_2, which is a known inducer of angiogenesis and inhibitor of apoptosis (Mutoh et al, 2006). Cyclooxygenase-2 proteins are overexpressed in 80% of human colorectal cancers, whereas expression in normal tissue is negligible (Sinicrope, 2004). Resveratrol (30 μM) was shown to reduce the activity of lipopolysaccharide-induced COX-2 in RAW 264.4 macrophages treated for 18 hours (Tsai et al, 1999) and in 184 B3/HER mammary cells following 30 minutes treatment (Subbaramaiah et al, 1998) and reduce PGE2 levels in the intestine of Min mice fed 90 mg (394 μmol) resveratrol/kg in the diet for 7 weeks (Ziegler et al, 2004). Resveratrol at 25 uM also decreased the activity of ornithine decarboxylase in Caco-2 cells (Schneider et al, 2000). Ornithine decarboxylase is a key enzyme in polyamine biosynthesis and is overexpressed in colorectal cancers (Nemoto et al, 2005).

In short, a variety of mechanisms may account for the chemopreventive activity of resveratrol. There is much that is still unknown and new advances in research technologies may provide the needed tools to fully realize the anticancer potential of resveratrol. Resveratrol may orchestrate changes at the cellular level that might be different depending on the tissue and/or chemical form and concentration of resveratrol. It is also possible that resveratrol may act synergistically with other polyphenolic compounds in plant-based foods to provide chemopreventive benefits. There is little doubt that this is a very active area of research and will continue to be so for the near future.

In our studies, azoxymethane was used to induce aberrant crypt foci lesions in the colon of CF-1 mice. Azoxymethane is converted to methylazoxymethane (MAM) by the hydroxylation of methyl groups catalyzed by CYP2E1 in the liver and extrahepatic organs

(Sohn et al, 2001). It is proposed that MAM is conjugated with glucuronic acid in the liver and is transferred to the intestine by the bile duct (Fiala, 1975; Fiala, 1977). Glucuronic acid conjugates can be hydrolyzed by bacterial β-glucruonidases to free methylazoxymethane, which spontaneously breaks down to yield methyldiozionium ions that can methylate DNA. Subsequently, the methyldiozionium ions would act directly at the site of the colonic mucosa resulting in the formation of aberrant crypt lesions and eventually, tumors.

Since we were interested in determining the chemopreventive activity of resveratrol-glucoside (piceid) in transgenic alfalfa, diets containing the alfalfa were administered to CF-1 mice three days after final AOM injections, ensuring that any effect observed in the colon of these mice on ACF development was not the result of an inhibition of AOM metabolism in the liver, because resveratrol can inhibit the activity of CYP2E1. The mechanism of resveratrol-induced inhibition of ACF was not explored in our study, but the ones proposed above in other studies could explain the activity we observed and report on later in this dissertation.

Bioavailability of resveratrol

Studies on the biological distribution of a proposed chemopreventive agent are necessary in order to examine the potential clinical value of that agent. Different research approaches have been applied in studying the bioavailability of resveratrol. Resveratrol bioavailablity has been examined in humans, laboratory rodents, *ex-vivo* intestinal preparations, and in human hepatic and colon human cell assays. Most of the bioavailability studies with resveratrol have been conducted using the *trans*-3,5,4'-hydroxystilbene isoform of resveratrol. Very few bioavailability studies have included piceid, and no studies could be located where other isoforms of resveratrol are examined.

Pharmakinetic studies reveal that resveratrol is rapidly absorbed following oral administration. Peak plasma levels of resveratrol (2-3 μM in mice and 1 μM in rabbits and rats) were reached within 5 minutes post-dose administration in animals fed 20 mg (88 μmol) *trans*-resveratrol/kg body weight (dissolved in 0.5 ml ethanol:saline 1:1) via gavage (Asensi et al, 2002). Plasma concentrations rapidly declined to levels below 0.1 μM in all animals after 60 minutes. Resveratrol reached a concentration of 42.8 ± 4.4 μM in the plasma of mice that received an i.p. injection of resveratrol at 88 μmol/kg body weight, but had a short half life of 14.4 minutes. Detected resveratrol levels in the brain, lung, liver and kidney were very low; all being under 1 nmol per gram of fresh tissue. When resveratrol was administered in the drinking water at 23 mg (100 μmol)/l to mice for 10 days, plasma concentrations of resveratrol were much lower than achieved with gavage (0.075 ± 0.025 μM). However, resveratrol inhibited the hepatic metastatic invasion of B16 melonoma cells that were injected into the spleen of mice prior to feeding the mice with resveratrol orally via gavage 88 μmol/kg body weight, twice daily) or drinking water 100 μmol/l). These findings illustrate that resveratrol may have very potent activity at specific tissues.

Peak plasma levels of resveratrol were reached rapidly in another study where Sprague-Dawley rats were administered oral (50 mg (219 μmol)/kg body weight in a solution of β-cyclodextrin) and intravenous (15 mg (66 μmol)/kg body weight in 0.2% saline) doses of *trans*-resveratrol-aglycone (Marier et al, 2002). In this study, plasma was collected over a longer time (from 0-12 hours post dose administration). The plasma concentrations of resveratrol-aglycone rapidly declined during the first two hours after dose administration with a mean elimination half-life of 0.13 ± 0.02 hours after i.v. administration and 0.29 ± 0.1 hours after oral administration. However, a sudden increase in plasma concentration

occurred 4 to 8 hours after drug administration, with a noted increase in glucuronide conjugates of resveratrol. Resveratrol-glucuronide plasma concentrations remained greater than 10 μM 8 hours out from the initial dose administration, whereas resveratrol-aglycone dropped off from a peak concentration of about 8 μM within minutes after treatment to levels below 1 μM after 2 hours. Enterohepatic recirculation of resveratrol was confirmed in a bile duct, rat linked model where resveratrol-aglycone was administered to bile-donor rats whose bile ducts were surgically linked via catheters to the duodenum of bile-recipient rats. These studies indicated that resveratrol is rapidly absorbed and circulates to the liver where it undergoes phase-II metabolism before being reintroduced into the intestine via the bile duct.

Vitrac et al. reported the tissue distribution over time of ^{14}C-*trans*-resveratrol-aglycone orally distributed to mice at a dose of 7.4 kbeqcuerels (0.2 μcuries) ^{14}C-*trans*-resveratrol-aglycone (5 mg (22 μmol)/kg body weight) by gastric intubation (Vitrac et al, 2002). The radioactivity in the blood 1.5 h post dose administration was low and did not increase throughout the 6 hour time-course of the experiment. However, earlier time points were not examined. Radioactivity was detected at high levels in the urine and bile at all time points. After 3 hours, radioactivity was detected in all organs and tissues detected, including the colon, brain, heart, liver, spleen, lung and testis. The highest levels were detected in the duodenum. To confirm that resveratrol accumulated in tissue, microutoradiography on the liver and kidney were performed. ^{14}C-*trans*-Resveratrol-aglycone along with glucuronic acid and sulfonoconjugates were identified in these organs.

Recently, positron emission tomography was used to visualize the biodistribution of a ^{18}F-labelled resveratrol derivative, 3,5-dihydroxy-4'-[^{18}F]-fluoro-*trans*-stilbene, in Wistar rats

injected intravenously with the compound (Gester et al, 2005). There was a rapid clearance of radioactivity from the blood, with levels at 5 minutes (0.15% of dose given) approaching the final concentrations measured after 60 minutes. The majority (37% of injected dose) of the radioactivity was detected in the urine of rats 60 minutes after they were injected with the isotope derivative at a single dose of 1.5 MBq [^{18}F] in 0.5 ml saline with 2% ethanol. Extensive uptake of resveratrol in the liver and kidney was suggested by the high levels of radioactivity measured in these tissues (12.6% and 5.6%, respectively). High levels of radioactivity (31% of dose) were also retained in the intestine 60 minutes after treatments, indicating that resveratrol can accumulate in this tissue.

Circulatory appearance and disappearance as well as tissue distribution of tritium-labeled resveratrol was assayed in Sprague-Dawley rats in a recent study (El-Mohsen et al, 2006). In this study, resveratrol was administered by gastric gavage at a dose of 50 mg (219 µmol)/kg body weight in a mixture with 1.85 MBq [^{3}H] to the rats. Biological samples were collected at 2 and 18 hours post-gavage, and tissue (brain, lungs, heart, liver and kidney) concentrations of resveratrol were determined using HPLC equipped with a diode array detector, whereas major metabolites of resveratrol were identified in the plasma and tissue using a mass spectrometer detector in tandem with fragmentation of trapped ions (MS/MS). Almost 90% of the resveratrol administered was absorbed in the rats over 18 hours post-gavage after accounting for losses in the feces. Besides the gastrointestinal tract, which retained 76% of the radioactivity administered, the highest tissue levels of radioactivity 2 hours post-gavage were obtained in the liver and kidney, which each contained less than 1% of the initial dose. Radioactivity in both the liver and kidney at 18 hours after treatment declined to 10 and 25% of the levels obtained at the 2 hour time point. However, the brain

retained 43% of the levels detected at 2-hours at the 18-hour time point, indicating that resveratrol may be retained in this tissue for a longer period. After 2 hours, resveratrol-glucuronide was detected as the only form of resveratrol in the tissues and in the plasma. Plasma concentrations of resveratrol-glucuronide reached 7 μM at 2 hours and could not be detected at the 18 hour time point. At 18-hours, resveratrol-glucuronide remained the major metabolite detected in the liver, but resveratrol-aglycone was the major form of resveratrol measured in the liver, heart, lungs and brain. This later observation would suggest that these tissues possess β-glucuronidase activity at significant levels. However, the concentrations of resveratrol-aglycone measured in these tissues at 18 hours was less than 0.2 nmol/g of tissue in all cases. Concentrations of resveratrol or identification of resveratrol metabolites in the colon or small intestine was apparently not determined in this study as these values were not reported.

The identification of phase-II metabolites of resveratrol in the plasma was the focus of a study in which male Wistar rats were fed daily doses of resveratrol-aglycone at 50 mg (219 μmol)/kg body weight or 300 mg (1.3 mmol)/kg body weight via the diet for 8 weeks (Wenzel et al, 2005). At the end of this treatment period, total resveratrol recovery in the urine and feces was 15% and 13%, respectively, in mice fed the 219 μmol/kg body weight dose, and 54% and 17% in mice fed the higher dose. No phase-I metabolites of resveratrol were detected in the plasma, urine or feces but various sulfate conjugates (resveratrol-3-sulfate, resveratrol-4-sulfate, resveratrol-3,5-disulfate, resveatrol-3.4-disulfate and resveratrol-3,5,4-trisulfate) as well as resveratrol-3-glucuronide were detected by high-performance liquid chromatography–diode array detection (HPLC-DAD) in the biological samples tested. Free resveratrol was not detected in the plasma or kidneys.

Phase-II metabolites of resveratrol were also reported to be the major isoforms of resveratrol in the circulation of humans administered ^{14}C-labeled *trans*-resveratrol (Walle et al, 2004). In this study, 0.1 mmole ^{14}C-labeled *trans*-resveratrol was given to six healthy adult humans at a 25 mg oral dose. Peak plasma levels of resveratrol (491 ± 90 ng/ml ≈ 2 μM) were reached within 1 hour after administration. As observed in the rodent study by Marier et al (2002), a second spike in plasma resveratrol concentrations (1.3 ± 0.3 nmol/ml), occurred at 6 hours post dose administration. After this point, plasma resveratrol concentrations rapidly declined and could no longer be detected. Resveratrol plasma concentrations also rapidly declined 1 hour after a 1.5 mg intravenous administration of resveratrol. No second resveratrol peak was observed following i.v. administration of resveratrol, though. Most of the resveratrol following the oral and i.v. doses was recovered in the urine (53.4-84.9 % and 42.3-83.2%, respectively). Resveratrol recovery in the feces following both i.v. and oral doses was highly variable (0.3-38.1% and 0.6-22.7, respectively). Free resveratrol could not be detected in any of the plasma samples. Instead, resveratrol was recovered as glucuronic acid and sulfate conjugates.

To compare the bioavailability of three polyphenolic compound constituents of red wine and the influence of beverage matrices, healthy male, adult human subjects were administered trans-resveratrol (25 mg (110 μmol)/kg body weight), catechin (25 mg (87 μmol)/kg body weight) or quercitin (10 mg (33 μmol)/body weight) in vehicles consisting of white wine, grape juice or vegetable juice (Goldberg and Soleas, 2003). Blood and urine samples were collected at four intervals over the first four hours after consumption. The vehicle matrices had no effect on the serum profiles of resveratrol throughout the times analyzed. In each case, peak serum levels of resveratrol were reached at 30 minutes with

total resveratrol concentrations maxing at approximately 2 μM. The serum concentrations of free resveratrol in all cases were a small fraction of total concentrations (1.7 to 1.9%). Similarly, the absorption of catechin and quercitin were not affected by the liquid matrices. Resveratrol was the most efficiently absorbed compound of the three, judged by the peak serum concentration, area-under-the curve and urinary excretion during the 4- hour period (16-17% of dose consumed). These results suggest that alcohol does not enhance the absorption of resveratrol-aglycone.

Transport and uptake models have been established using whole liver and small intestine tissues as well as intestinal and heptatic cells. Rapid glucuronidation and sulfation of resveratrol following incubation with human liver microsomes have been documented (De Santi et al, 2000a; De Santi et al, 2000b). Both *trans*- and *cis*-resveratrol were subject to glucuronidation, which was region and stereoselective, and resulted in the formation of 3′-O and 4′-O glucuronides. This is in agreement with the human data showing that 3′-O- and 4′-O- glucuronides are the major metabolites of resveratrol in the blood (Wang et al, 2004 and reviews above).

Using an isolated preparation of luminally and vascularly perfused rat small intestine, Andlauer et al. (2000) demonstrated that phase-II metabolism of resveratrol occurs in the small intestine. In this study, luminal media passing through the small intestinal preparations consisted of 28, 34 or 57 uM resveratrol. Following a 30 minute perfusion period, only 20.5% of the resveratrol administered was recovered on the vascular side, as detected by HPLC/MS. The majority of the resveratrol absorbed was conjugated to yield resveratrol glucuronide (82% of resveratrol absorbed). Lesser amounts of resveratrol-aglycone (3.6%) and resveratrol sulfate (0.3%) were detected in the vascular effluent. Another group, using a

similar rat small intestine single-perfusion model, showed that the major metabolite detected on vascular side of the jejunm and ileum was the glucuronide conjugate of resveratrol (96.5% of the amount of resveratrol absorbed) following a 90- min period where resveratrol at a concentration of 200 μM was perfused through the lumen of the isolated intestinal sections (Kuhnle et al, 2000).

A cell-based assay using Caco-2 cell monolayers was used to study the transport of resveratrol (5-40 μM) in the intestines (Kaldas et al, 2003). When resveratrol was incubated with the cells at the lower concentration of 5 μM, apical efflux of resveratrol was favored. However, basolateral efflux of resveratrol was favored at higher doses of 20 and 40 μM. The transport rate of resveratrol doubled from doses 20 to 40 μM, with the recovery of resveratrol-3´-O-sulfate decreasing by more than two-fold at 40 μM, suggesting that the saturation of phase-II metabolism may, at least, partially explain the increase in basolateral transport of resveratrol at higher doses. The transport of resveratrol-3´-O-sulfate to the apical side of the cell monolayer system at lower resveratrol doses indicates that the multidrug-resistance protein may play a significant role in transporting intracellular resveratrol back out into the lumen following phase-II metabolism of absorbed resveratrol. In spite of this, the intracellular accumulation of resveratrol was extensive in Caco-2 cells, reaching as high as 40-fold compared to the surrounding buffers.

The Caco-2 monolayer system was used in another study to further probe the transport mechanism of resveratrol in the intestine (Li et al, 2003). The apical and basolateral efflux of resveratrol was similar in all concentrations examined (5-250 μM), indicating that resveratrol-aglycone is passively transported across the epithelium of the intestine. Furthermore, this study did not support a role for P-glycoprotein or the multi-drug resistance

associated proteins (MRP) in the apical transport of resveratrol as the addition of specific inhibitors to these proteins did not affect the transport of resveratrol across Caco-2 monolayers. Resveratrol-3-glucuronide and resveratrol-3-sulfate were identified as the major metabolites of resveratrol in both the apical and basolateral fractions of a monolayer cultured with 200 uM resveratrol.

Resveratrol-3-o-glucuronide and resveratrol-3O-sulfate, along with resveratrol-4-o-glucuronide were identified as metabolites produced in Caco-2 cells exposed to resveratrol in a monolayer system in another report (Maier-Salamon et al, 2006). In this study, Caco-2 cells were treated with a variety of doses (10-200 uM). The apical to basolateral rate of resveratrol was 12 x 10^{-6} cms^{-1}, which indicates that resveratrol is rapidly absorbed in these cells and supports the data gathered by the previous studies in humans and animals showing that resveratrol is rapidly absorbed within 30-60 minutes following oral administration. As with the study by Li et al (2003), the basolateral to apical rate for resveratrol was similar, but slightly less than the reverse rate, indicating that a basolateral efflux is marginally favored. However, the basolateral efflux of resveratrol was increased by 22% in cells treated with 10 uM resveratrol and 5 μM cyclosporine, an inhibitor of the muti-drug resistance protein (MRP) and p-glycoprotein. The presence of 40 μM verapamil, a specific inhibitor of p-glycoprotein, had no effect on resveratrol efflux, indicating that the MRP plays a role in the apical efflux of resveratrol, contradicting the findings of Li et al, but supporting the hypothesis generated by Kaldas et al (2003). The basolateral efflux of both resveratrol monoglucuronides vs. treatment concentration followed a classic Michaelis-Menton kinetics pattern as the efflux of these metabolites increased linearly at lower concentrations but reached a saturation point at higher doses. However, biotransformation of resveratrol to

resveratrol-3´-O-sulfate was strongly inhibited in cells treated with higher concentrations of resveratrol (200 μM). Remarkably, the intracellular concentrations of *trans*-resveratrol remained at about 61% of the applied dose across the treatments applied, with the concentrations ranging from 370 pmoles/cm^2 of the cell layer at a dose of 10 uM to 37,200 pmoles/cm^2 at a dose of 200 μM.

Bioavailability of piceid

In comparison to resveratrol-aglycone, much less is known about the bioavailability of piceid. The bioavailability of resveratrol via a grape extract, which consisted mostly of piceid, was examined in humans (Meng et al, 2004). Grape powder that contained 38 umol total resveratrol/kg dry weight was mixed into a juice (18% aqueous solution of grape preparation – juice contained 0.16 mg total resveratrol/ml) and administered to adult subjects at a single volume of 200, 400, 600 or 1200 ml (corresponding to 32-192 mg total resveratrol). Resveratrol could not be detected in the plasma or urine of subjects given 400 or 600 ml of the grape juice. The cumulative excretion of resveratrol in the urine after drinking 1,200 ml of grape juice was only 5% of the dose administered. Furthermore, resveratrol was detected only following hydrolysis with β-glucuronidase. Resveratrol-aglycone was not detected in the plasma even at the higher doses examined. The resveratrol recovery in the urine was one-tenth of what was obtained in subjects administered pure resveratrol at a similar dose (1.95 mg per 65 kg weight). Since most of the resveratrol in the juice consisted of glucosides, this study suggested that the bioavailability of resveratrol-glucosides is very limited compared to resveratrol-aglycone.

Recently, a group reported a new HPLC method to extract and measure piceid in biological tissues (Lv et al, 2006). This report featured a study were piceid was orally

administered to male Wistar rats at a dose of 50 mg (128 µmol)/kg body weight in an aqueous vehicle consisting of 0.5% sodium carboxymethylcellose (it was not specified whether this was achieved using gavage or via drinking water). Plasma was collected 5 to 240 minutes post dose administration. A separate study was conducted using the same dose of piceid in order to assess piceid levels in tissues following 10, 30 and 120 minutes post dose administration. All sample extracts were analyzed using HPLC with a diode array detector. Peak plasma concentrations of piceid (0.364 ± 0.151 µg/ml, equivalent to 0.9 µM piceid) were reached 20 minutes after treatment and gradually decreased to undetectable levels after 240 minutes. Piceid was detected in all of the tissues examined, but were at the highest levels in the stomach and small intestines. The levels in these tissues were over 0.3 µmol piceid/g tissue at 2 hours and declined to levels under 13 nmol/g after 18 hours. Besides the gastrointestinal tract, the highest concentrations of piceid were observed in the spleen and lung, with concentrations reaching around 25.6 nmol/g tissue after 10 minutes. Levels in the brain, liver, testis and kidney were around 5 nmol/g tissue at 10 minutes. After 120 minutes, piceid levels declined to less than 2.5 nmol/g tissue in all of the samples examined. Collectively, these data imply that piceid is bioavailable, but is rapidly eliminated in rats. However, none of the biological samples were treated with β-glucuronidase or sulfatase enzyme prior to analysis, which leaves open the possibility that piceid may be metabolized to other forms. Unfortunately, a side-by side comparison with resveratrol-aglycone was not conducted.

Comparisons between piceid and resveratrol-aglycone intestinal transport have been made using the Caco-2 monolayer cell assay. Henry et al (2005) reported that resveratrol-aglycone was absorbed across the apical membrane of Caco-2 cells more rapidly and in

higher amounts than trans-piceid. The recovered intercellular concentrations of resveratrol-aglycone remained at least three times greater than that of piceid in Caco-2 cells exposed to equal molar concentrations of the two compounds (300 uM) in all time points examined (0-30 minutes). The presence of phlorzin, an inhibitor of the sodium-glucose cotransporter-1 (SGLT-1), did not affect the absorption of resveratrol-aglycone across the Caco-2 monolayers, which further supports the data suggesting that resveratrol-aglycone is passively absorbed in the intestine. The active transporter SGLT-1, however, does seem to play an important role in the intestinal absorption of piceid as the addition of phlorizin inhibited the cellular uptake of piceid in Caco-2 cells.

Lactase phlorizin hydrolase (LPH), an enzyme located at the brush border membrane of the small intestine that is responsible for lactose hydrolysis, can deglycosylate certain flavonoid 3′- and 4′-glucosides, such as quercitin-3'-glucoside and quercitin-4′-glucoside (Day et al, 2001; Sesink et al, 2002) and may play an important role in the small intestinal absorption of other polyphenolic-glucosides. Since LPH is located outside of the epithelial cells along the enterocyte membrane, the deglycosylation of glycosylated compounds can occur in the lumen before the compound is absorbed across the enterocyte membrane. In addition to LPH, other β-glucosidase enzymes have been identified in the small intestine cytosol and can hydrolyze glycosylated flavonoids to their respective aglycones (Day et al, 2001). However, in order for any polyphenolic-glucoside to be a potential substrate for cytosolic-β-glucosidases, the compound would have to be initiallytransported across the enterocyte membrane of the intestine.

The role of LPH in the intestinal metabolism of piceid was explored in a recent study by Henry –Vitrac et al (2006). The transport permeability coefficient of 100 μM piceid across

Caco-2 monolayers was 10.5 X 10^{-6} ± 0.1 cm/s for the apical to basolateral direction and 15.3 x 10^{-6} ± 6.6 cm/s in the basolateral to apical direction. The basolateral to apical rate was significantly greater than the reverse rate, but the rate for the apical to basolateral flux measured in this system predicts that piceid should be efficiently absorbed in the gastrointestinal tract. In Caco-2 cells that were seeded to 6-well plates and treated with 100 uM piceid, evidence of piceid deglycosylation was obtained as the intracellular concentration of *trans*-resveratrol-aglycone measured increased in a time-dependent, linear fashion to a maximum concentration of 4.5 ± 0.1 pmol/cm^2 after 24 hours oftreatment. To determine if the recovery of the aglycone was the result of a catalyzed event by LPH or cytosolic-β-glucosidases (CBG), the intestinal mucosa of Wistar rats was scrapted and extracted to obtain fractions that contain LPH or CBG. Resveratrol-aglycone was detected following piceid incubation with both extracts. The recovery of resveratrol-aglycone was inhibited by 93% in cells incubated with LPH and by 69% in cells with CBG by 2 mM D-gluconolactone, an inhibitor of both cytosolyc and membrane-bound β-glucosidases, which reveals that piceid can be a substrate for both types of cellular β-glucosidases. Resveratrol-3-O and resveratrol-4-O-glucuronide were identified as the major resveratrol metabolites in Caco-2 cells. Treatment of cells with 50 uM of crysin, a known inducer of UDP-glucuronosylftransferases of the 1A1 family (UGT1A1), resulted in an increase of both resveratrol-monoglucuronides.

In summary, the current collective data raise some interesting questions on the potential role of resveratrol-aglycone as a chemopreventive or therapeutic agent at target tissues beyond the gastrointestinal tract. The bioavailability studies indicate that resveratrol, following an oral dose, is rapidly adsorbed in the upper gastrointestinal tract where it

undergoes extensive phase-II metabolism before transferring to the systemic circulation. In the liver, resveratrol undergoes a second pass of phase-II metabolism and then is re-circulated to a variety of tissues, including the heart, lung, brain, prostate and kidney. As reported above, there is some evidence that tissue accumulation of resveratrol occurs at these sites. However, the bioactivity of glucuronide or sulfate conjugates of resveratrol, which by all indications are the predominant forms of resveratrol reaching these tissues have not been examined. Furthermore, the role of β-glucuronidases or sulfatases at these sites remain unknown in regards to resveratrol metabolism as some studies indicate that resveratrol can be found in the native aglycone form at these sites.

Sor far, no studies of the accumulation of resveratrol in the colon of an animal model following an oral dose were found in the literature. Although resveratrol seems to be absorbed mainly in the upper g.i. tract, some of the compound could remain in the intestinal lumen and reach the colon. One study reported that phenolic degradation products of resveratrol could not be detected in the urine or tissues of Sprague-Dawley rats administered 50 mg/kg body weight of [3H] trans-resveratrol 2 or 18 hours post-gavage following LC/MS-MS analysis of tissue (El-Mohsen et al, 2006). Since resveratrol glucuronide and sulfate metabolites could be examined in the biological samples, this would suggest that resveratrol-aglycone is not degraded to other compounds, but this has not been confirmed in humans. Studies in Caco-2 cells and radiotracer readings in the gastrointestinal tract of rodents administered radiolabeled versions of resveratrol suggest the possibility that resveratrol may accumulate in the colonic enterocytes once absorbed. The bioavailability studies in rodents also suggest that resveratrol-glucuronide or sulfate metabolites can be reintroduced into the intestinal lumen via the liver from the bile duct. In the colon, β-

glucuronidase and sulfatase activity expressed by the gut microflora could hydrolyze the conjugated metabolites (Aura et al, 2002), releasing free resveratrol-aglycone at the site of the colon.

With piceid, the picture is much less clear. The bioavailability studies indicate that piceid can be absorbed in the upper g.i. tract via an active process that may involve the SGLT1 or may be deglycosylated by LPH and then passively absorbed as resveratrol. It is not known if this occurs in humans or animals, since this has not been demonstrated in either yet. The one study by Lv et al (2006) indicates that piceid may reach the circulation intact, but the plasma concentrations of the compound were very low, and little is known about the biological activity of piceid relative to resveratrol-aglycone. Also, it is not known if the metabolic fate of the absorbed piceid is the same as resveratrol-aglycone. Bilary excretion may be a major pathway for resveratrol-aglycone following phase-II metabolism in the liver, which would provide an alternative pathway to the colon (Marier et al, 2002). This could be the same with piceid. Alternatively, renal excretion may be the terminal fate of piceid absorbed in the small intestine. Piceid that passes through the small intestine, unabsorbed or metabolized, would reach the colon where it could be absorbed or be a substrate for microbial β-glucosidases.

One of the goals of this research was to examine the colon bioavailability of piceid in diet preparations containing piceid-accumulating transgenic alfalfa with and without β-glucosidase activity. Additionally, we determined whether piceid is a substrate for LPH in the small intestine by comparing the recovery of piceid in the mucosal and serosal tissues of isolated, everted intestinal mouse sacs incubated in piceid in the presence and absence of a lactase-specific inhibitor. To our knowledge, our studies are the first to examine the role of

LPH in the intestinal absorption of piceid and compare its absorption with that of resveratrol-aglycone *in-vivo*. Our studies are also unique in that we link the colon bioavailability of piceid and resveratrol-aglycone to the inhibition of azoxymethane-induced aberrant crypt foci in this tissue.

Genetic-engineering strategies to increase resveratrol availability in plant-based foods

Given the limited number of identified natural and processed consumable sources of resveratrol and the low levels of resveratrol in these sources, it is unlikely that desired biological endpoints could be achieved from normal dietary consumption of resveratrol alone. Instead, strategies will have to be employed to either increase yields of resveratrol in present sources, introduce resveratrol into additional plants, or concentrate resveratrol into an extracted or purified, marketable product. Genetic engineering strategies could be developed to achieve each of these goals. The second and remaining portion of this review will focus on the potential promise and limitations of using transgenic approaches to enrich the availability of resveratrol and related compounds in dietary sources to address human health concerns.

Definition of "genetically-modified" and advantage over traditional breeding

The terms "genetically-enhanced, genetically-engineered, genetically-modified" and "transgenic" collectively describe an organism that is generated using recombinant DNA technology (Celec et al, 2005). Genetic diversification in plants has been achieved for thousands of years through traditional breeding practices (Diamond, 2002). However, genetic engineering technology offers the advantage over traditional plant breeding approaches in that the generation of useful phenotypes is not limited by the gene pool of existing plant germplasms. Additionally, there is growing concern that traditional plant

breeding practices may have resulted in the selection of crops with improved yields at the expense of decreased nutritional quality (Morris and Sands, 2006). In the last decade and a half, advances in molecular biology have made it possible to selectively introduce novel genes into crops from a virtually unlimited number of sources. As the result of this, new crops with disease-, pesticide- and insect- resistant traits; increased tolerance to environmental stress; and enhanced nutritional composition have been developed (Singh et al, 2006; review).

Plant transformation techniques

Currently, two methods are widely used to genetically transform plants (Tourte, 2005). One method involves the use of the bacterium *Agrobacterium tumefaciens,* which can insert a plasmid into wounded plants. Laboratory strains of *A. tumefaciens* employ a binary plasmid that encodes several genes required for the transfer of the plasmid into the plant cells and engineered expression cassettes that can contain genes from virtually any source imaginable. The second method widely used is the biolistic method, which involves propelling microprojectiles, usually of tungsten or gold, coated with DNA into targeted plant cells. Traditionally, the *Agrobacterium* method has been applied to the transformation of dicotyledonous plant species, whereas the biolistic method has been employed for monocotyledonons species. However, in recent years, *Agrobacterium*-mediated techniques have been established for the transformation of cereals and rice, crops that were once deemed impossible to transform with this method.

Scope of GM market

The estimated global area planted with genetically-modified crops for 2005 was 90 million hectares, equivalent to 222 million acres, grown by 8.5 million farmers in one of 21

countries, up from 17 countries reported in 2004 (http://www.iaaa.org). Since the commercial introduction of GM crops in 1996, the average growth in area has been more than 10% per year (Dunwell, 2005).

Of the global area planted, about 99% of GM crops are grown in six countries: the United States, Argentina, Canada, Brazil, China and South Africa (Dunwell, 2005). The United States, by far, leads all nations in the production of commercial GM crops. In 2005, 123 million acres of land in the U.S. was utilized for GM crop production. The leading GM crops are currently soybean, corn, cotton and canola. Based on the prevalence of soybean and corn in the U.S. food system, the Grocery Manufacturers of America estimates that 70% to 85% of processed foods in the U.S. contain one or more ingredients derived from GM plants (Bren, 2003). Thus, it is highly likely that every adult residing in the U. S. has consumed at least one GM product in their lifetime.

Plant-transforming technologies have improved in efficacy since the first GM crop became commercially available in the U.S. in 1994, and applications have been developed to extend the technology to a number of plant species (Celec et al, 2005). Genetically-modified crops that express tolerance to herbicides, insect-resistance or both of these traits stacked represent the GM market today and account for the vast majority of acres devoted to GM crops in the world (Dunwell, 2005). There is strong evidence that these crops have had a positive impact on the environment and have significantly resulted in profits for farmers who have adapted their use. In 2000, it was reported that farmers in the United States who grew transgenic cotton used 2.4 million fewer gallons of fuel and 93 million fewer gallons of water (Falck-Zepeda et al, 2000). It is estimated that the total economic benefits to farmers in the U.S. who plant GM crops are around $1.5 billion per year. A recent report

suggests that biotech crops have reduced the environment footprint associated with pesticide use by 14% from the years 1996-2004 (Brookes and Barfoot, 2005). The economic benefits of GM crops are now being felt worldwide. South African and Chinese farmers growing transgenic insect-resistant cotton, for instance, achieve yield increases of more than 20% and cost savings of nearly 30% (Entine, 2006).

GM crop production to address nutrition and health discrepancies

Plants synthesize and accumulate a wide array of nutrients and other compounds that have health-promoting properties. A diverse, well-balanced plant-based diet, in theory, can supply all of the currently identified essential nutrients at all stages of the life cycle; the exceptions being vitamins B12 and D (Grusak and Della Penna, 1999). However, in practice, very few individual plants supply the daily recommended intakes of any micronutrient in a reasonable serving, and the bioavailability of some micronutrients is very limited in plant-based foods. Furthermore, many people do not consume a diet diverse enough to obtain the full range of health-benefiting attributes that can be gained from these foods. In many developing countries, people spend 70% of their income on food, with the majority of the energy requirments being supplied in the form of staple crops such as rice, wheat and maize, which have traditionally been poor sources of some macronutrients and many micronutrients (Bouis et al, 2003). To augment the consumption of various plant-derived nutrients and nutraceuticals by people around the world, biotechnology is being applied to enhance the nutritional composition of crops (Bouis et al, 2003; Yan and Kerr, 2002; Grusak and Della Penna, 1999). Examples of these crops include rice which accumulates β-carotene, a precursor of vitamin A (Ye et al, 2000); phytase (Holm et al, 2002) and ferritin-rich rice (Lucca et al, 2001) with improved biological bioavailabilty of iron; a soybean with a higher

percentage of oleic acid and decreased levels of polyunsaturated fatty acids (Kinney and Kowlton, 1998), canola which produces omega-3 fatty acids (James et al, 2003), a potato with increased biosynthesis of lysine (Seveneir et al, 2002), tomato with elevated biosynthesis of β-carotene (Romer et al, 2000) and soybean with increased levels of α-tocopherol (Cahoon et al, 2003).

Many compounds have been identified in plants that may have health promoting or disease preventing properties beyond the mere provision of basic nutrition (Lampe, 2003), (Tsuda et al, 2004) and (Baliga et al, 2005). Some of the phytochemical compounds produced as secondary metabolites by plants may be of value for health as well as agronomical purposes, because their presence can improve crop yields and provide health beneficial properties to humans (Verpoorte et al, 2000; Rao and Ravishanker, 2002; Caspell and Christou, 2004). A number of phytochemical polyphenols are isolated from plants and are already commercially available as chemicals for use as drugs, dyes, flavors, fragrances and insecticides (Verpoorte et al, 2000). Some of these compounds, such as the flavonoids, play an important role in the plant's defense against UV-B light exposure and pathogens, pollen development and fertility, and flower and fruit color development (Woo et al, 2005). These attributes have been exploited in some plants through genetic-engineering strategies to produce plants with greater growth yields and desirable physical traits. From a nutritional point of view, flavonoids are of particular interest since many of these compounds have been suggested to protect against oxidative stress, coronary heart disease, certain cancers and age-related diseases (Harborne and Williams, 2000). Examples of GM plants that have been introduced include alfalfa that produces genistein glucoside (Deavours and Dixon, 2005) and greater yields of anthocyanins and proanthocyanidins (Ray et al, 2003), tobacco that

expresses a phenylalanine ammonia-lyase gene from the Chinese herb *Astragalus membranaceus* and accumulates higher levels of quercetin (Lui et al, 2006), and tomato which expresses maize transcription factor LC and C1 genes and produces flavonoids in the fruit flesh in addition to the peel (Bovy et al, 2002). One of the most remarkable successes in recent years has been the production of a tomato which overexpresses petunia chalcone isomerase and exhibits an 80-fold increase in the content of flavonoids in the fruit peel (Muir et al, 2001). In a recent study, Rein et al. assessed the potential benefits of this transgenic tomato on cardiovascular health using human C-reactive protein transgenic mice (CRPtg) as test subjects (Rein et al, 2006). The transgenic tomatoes used in this study accumulated high levels of quercetn-3-rutinoside (12120 ± 760 mg/kg dry peel) and kampferol-rutinoside (1985 ± 118 mg/kg dry peel) among other flavonols and flavones. The peel of the transgenic tomato was incorporated into a standard rodent diet at a concentration of 4 g peel/kg of diet and fed to CRPtg mice which for 7 weeks. Both wild type and transgenic tomato peel reduced basal plasma human c-reactive protein levels in the mice by 43 and 56%, respectively. However, the reduction with the transgenic tomato was significantly higher than that with the wild-type tomato peel.

It is important to emphasize that none of the crops mentioned in this section are approved for commercial use and are currently at various stages of field trials. Scientific research is still needed to confirm the potential benefits and hazards of these crops.

Genetically-engineering plants for resveratrol accumulation

Stilbene compounds, such as resveratrol, are flavonoid-type phytoalexins formed on the phenylalanine/polymate pathway (Jeandet et al, 2002). The terminal enzyme involved in resveratrol synthesis, *stilbene synthase* (StS) (a.k.a. *resveratrol-synthase*), catalyzes the

decarbocylative condensation of a *p*-coumaroyl residue from *p*-coumaroyl-CoA with three C_2 subunits from maloynyl-CoA (Ruhmann et al, 2006). P-coumaroyl-CoA and maloynyl-CoA are shared precursors for other polyphenolic compounds in the flavanoid biosynthesis pathway that are synthesized in a series of reactions initiating with one catalyzed by *chalcone-synthase*. *Stilbene synthase* and *chalcone synthase* (CHS) belong to the polyketide synthase (PKS) superfamily since they catalyze common condensation reactions of *p*-coumaroyl CoA/cinnamoyl-CoA and three units from malonyl-CoA (Jeandet et al, 2002). Chromsomal DNA sequences show that StS enzymes from different plants share 70% identity to each other and many CHS enzymes, suggesting that StS evolved from CHS during plant evolution (Tropf et al, 1994). *Stilbene-synthase* and CHS catalyze different cyclization reactions to produce resveratrol and naringenin-chalcone, respectively, from the same precursors of p-coumaroyl CoA and three molecules of malonyl-CoA. Naringenin-chalcone is a precursor of various classes of flavonoid compounds, including flavones, flavonols, catechins, deoxyflavonoids and anthrocyanins (Schijlen et al, 2004).

Expression of StS is induced in plants by fungal infection and abiotic stimuli such as UV light and ozone (Jeandet et al, 2002). The exception seems to be in grape seedlings where StS is constitutively expressed (Sparvoli et al, 1994). As mentioned earlier, resveratrol is found in a variety of plant species, but in a limited number of varieties consumed by humans. The introduction of resveratrol into novel plants through genetic-engineering is theoretically an attractive plan since resveratrol is a proposed phytoalexin and has many purported health benefits (reviewed earlier). Therefore, such crops should offer both producer-oriented and consumer-oriented benefits. Furthermore, the introduction of a single gene is sufficient to synthesize resveratrol in plants, unlike the scenario faced with other

flavanoid compounds, where multiple genetic insertions may be required (Jeandet et al, 2002).

To date, StS has been ectopically expressed in tobacco (Hain et al, 1993), rice (Stark Lorenzen et al, 1997),barley and wheat (Leckland et al, 1998), tomatoes (Thomzik, 1997), kiwi (Kobayashi et al, 2000), apple (Sparvoli et al, 1994; Ruhmann et al, 2006), white poplar (Giorcelli et al, 2004), arabidopsis (Yu et al, 2006) and rapeseed (Husken et al, 2005). Agrobacterium-mediated, biolistic and protoplastic techniques have all been employed in transforming crops to express StS from either grapevine or peanut sources. In all experiments, the expression of single StS genes was optimized by using heterologous promoters and enhancer elements. The introduction of StS has resulted in plants that express resistance to a variety of pathogens, including *Pyriculeria oryzoa* in rice (Stark Lorenzen et al, 1997), *Botrytis cinerea* in wheat and barley (Leckland et al, 1998), *Dhytophthora infestans* in tomato (Thomzik, 1997) and *Oidrum tuckeri* in wheat (Leckland et al, 1998). In kiwi and white poplar, transformed plants did not express disease resistance against the pathogens examined, which are the exceptions to RS-transformed plants reported in the literature (Kobayashi et al, 2000; Giorcelli et al, 2004).

In the cases where the presence of resveratrol or derivatives thereof were examined in transformed plants using either HPLC units equipped with a diode array or mass spectrometer detector, resveratrol was demonstrated to accumulate almost exclusively in the form of *trans*-resveratrol-3-glucoside (piceid), suggesting that endogenous modification of resveratrol by a glycosyltransferase occurs. In arabidopsis, piceid was predominately identified in the *cis*- confirmation (Yu et al, 2006), whereas both trans-piceid and *trans*-resveratrol-aglycone were identified in transformed tomato (Thomzik, 1997).

The accumulation of piceid has been reported to vary widely between plant species and cultivars genetically-modified to overexpress StS. Transgenic plant lines have been reported to accumulate as much as 400 ug piceid/g fresh weight of piceid, as in the case of tobacco (Fischer et al, 1997). In some plants, such as wheat and barely, piceid concentrations were very low in plants that contained only one copy of the StS gene, whereas higher yields and greater disease resistance was obtained in plants expressing multiple copies of the gene (Leckland et al, 1998). However, piceid could not be detected in a transformed apple cultivar which expressed 6 copies StS, indicating that gene silencing can occur in some plants with multiple copies of StS (Ruhmann et al, 2006). Piceid accumulation can also be restricted to certain tissues and, in many cases, appears to be age-dependent. In the kiwi plant, piceid accumulation was much higher in younger leaves (182 ug piceid/ g dry leaf weight) than in older leaves (20 ug/g dry weight) (Kobayashi et al, 2000). In apple, piceid accumulation varied between cultivars (Ruhmann et al, 2006). Piceid accumulated in both the ripe and unripe fruit of the transformed "Holsteir Cox" line of apple but was detected only in the unripe, green fruit of the 'Elsteir' cultivar. Flavonol levels were slightly decreased in the transgenic fruits that accumulated piceid; the levels of other polyphenolic compounds analyzed did not seem to be affected by the introduction of the StS transgene.

Phytochemical analysis of transformed StS-expressing plants has rarely been carried out, but the data that exist strongly suggest that substrate competition between flavonoid and resveratrol biosynthesis can occur. In apple, overexpression of StS and the consequent accumulation of piceid was accompanied by lower levels of flavonols (Ruhmann et al, 2006). Overexpression of StS in tobacco resulted in male sterility and plants that produced faint pink flower petals, as opposed to the dark pink color observable in non-transgenic,

control tobacco plants (Fischer et al, 1997). This report suggested that the diminished flower pigmentation and male sterility was not the result of endogenous suppression of chalcone-synthase, but rather due to competition for 4-coumaroyl CoA and malonyl CoA substrates between resveratrol and flavaonol biosynthesis, since certain flavonols play an important role in plant pigmentation and sterility. Decreased fertility and altered growth patterns have been described in other plants transformed to accumulate specific flavonoids (Schijlen et al, 2004; review). Metabolic flux competition between endogenous pathways and ectopically introduced pathways can potentially pose a barrier in introducing a novel compound into plants. For example, substrate competition with the phenylpropanoid pathway leading to the accumulation of anthocyanins was proposed as the culprit in the failure to achieve high levels of isoflavonoids in transformed isoflavone-synthase-expressing, non-leguminous plant species (Yu et al, 2000). Consequently, the introduction of key enzymes involved in isoflavone synthasis into novel species has been met with little success beyond soybean plants and bacteria (McCue and Shetty, 2004; review).

Collectively, the contradictory results of these studies suggest that the introduction of StS into plants can lead to high accumulation of piceid and significant pathogen control, but the end result is not predictable and depends on the plant species and, in some cases, on plant age. It is conceivable that metabolic flux changes in flavanoid biosynthesis resulting from the overexpression of StS is the cause of the discrepancy in pathogen protection noted in certain transformed plants, because the introduction of RS into some plants may alter changes in defense systems involving other flavanoid compounds. However, this is purely speculative since the mechanism (s) behind the purported phyoalexin capabilities of resveratrol has not been entirely elucidated (Hammershcmidt, 1999). Studies with GM

plants reveal that one of the major considerations in developing a natural food product to produce higher yields of resveratrol is endogenous modification of resveratrol in the plant. Metabolic engineering of other plant species have also resulted in the accumulation of a glycosylated or methylated version of the flavonoid compound (Deavours and Dixon, 2005). Not much is known about the modifying enzymes and corresponding genes responsible for these endogenous modifications in plants.

Transgenic piceid-accumulating alfalfa

The transgenic alfalfa used in our studies was originally produced to protect the plants against root rot caused by the fungal pathogen *Phoma medicaginis*. The original alfalfa plants (*Medicago sativa* variety Regen SY) were transformed with a peanut (*Arachis hypogaea*) cDNA encoding resveratrol-synthase under the transcriptional regulation of the enhanced cauliflower mosaic virus 35S promoter (Hipskand and Paiva, 2000). As with most of the plants genetically-modified to synthesize resveratrol, the transformed alfalfa plants demonstrated resistance against a notable plant pathogen (*Phoma medicaginis)*. And, as with other plants that over-express RS, resveratrol accumulated as *trans*-resveratrol-3-*O*-glucoside (piceid) in the transformed alfalfa plants, as confirmed by high-pressure liquid chromatography (HPLC) and ^{1}H and ^{13}C-nuclear magnetic resonance (NMR) analysis. Piceid was detected at the highest concentrations in the youngest leaves (expressed as 0.5-20 µg resveratrol-equivalents/g fresh tissue, due to the lack of appropriate piceid standards available at the time of the study). Roots contained only trace amounts of piceid (<0.2 µg resveratrol equivalents/ g fresh tissue) as well as sprouts (Paiva, personal communication). Roots and sprouts accumulate high levels of flavanoids and isoflavones. However, the low concentrations of piceid in these tissues were not attributed to compentition between the

flavonoid and stilbene biosynthesis pathway, since chalcone-synthase mRNA levels were higher in these tissues relative to RS mRNA levels.

Alfalfa is a highly utilized crop because of its productivity and high feed value. It is a very rich source of protein (which constitutes 15-22% of the plant), vitamins and minerals (North American Alfalfa Improvement Conference (NAAIC), 2005). Alfalfa also contains numerous secondary metabolites such as saponins, flavonoids, tannins, carotenoids and tocols (Stochmal et al, 2001). Some of the major flavonoids identified in alfalfa include glycosides of apigenin, lutelin, tricin and chrysoeriol (Stochmal et al, 2001 a and b). Alfalfa is primarily used as a forage crop and is the fourth most widely grown crop in the United States behind corn, wheat and soybeans (NAAIC, 2005). In addition to its use in animal feeds, alfalfa is consumed by humans in the form of sprouts and occasionally as a tea or juice. Alfalfa offers many characteristics that make it an ideal crop for biotechnology applications. Its vegetative growth can be maintained for years; clonal propagation through stem cuttings can make batch-to-batch reproducibility of protein expression in the plant a highly feasible task; and modern varieties are capable of expressing high amounts of protein (D'Aoust et al, 2004).

We were interested in determining if the transgenic, piceid-accumulating alfalfa could provide any additional health benefits beyond those that can be obtained from normal, non-transgenic alfalfa. Specifically, we analyzed the bioactivity of the transgenic alfalfa in the colon against AOM-induced carcinogenesis in CF-1 mice as resveratrol has purported chemopreventive properties in the colon (reviewed earlier) and the Birt laboratory is experienced in working with this rodent model of colon carcinogenesis (Au et al, 2006).

The initial studies with transgenic alfalfa, as discussed in more detail in chapter 2, revealed that piceid may not be bioavailable. The primary focus of this thesis was to test the hypothesis that piceid from the transgenic alfalfa is not bioavailable and that additional strategies are required to liberate the more bioavailable, native resveratrol-aglycone compound from piceid making the transgenic alfalfa more bioactive against the formation of AOM-induced ACF lesions in the colon of CF-1 mice. The strategies employed in the studies included supplementing diets containing the alfalfa with exogenous β-glucosidase enzymes. The hypothesis was tested by examining the effect that these dietary preparations had on the formation of AOM-induced ACF in the colon of CF-1 mice and the resulting colonic and plasma concentrations of resveratrol following the administration of these diets in 5-week periods. The mechanism of the intestinal bioavailability of piceid was assessed by examining the substrate suitability of piceid for lactase phorizin hydrolase in the small intestine.

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CHAPTER 2: TRANSGENIC ALFALFA THAT ACCUMULATES PICEID (TRANS-RESVERATROL-3-O-β-D-GLUCOPYRANOSIDE) REQUIRES THE PRESENCE OF β-GLUCOSIDASE TO INHIBIT THE FORMATION OF ABERRANT CRYPT FOCI IN THE COLON OF CF-1 MICE

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Abbreviations: Aberrant crypt foci (ACF), *trans*-resveratrol-aglycone (Rag), Piceid = *trans*-resveratrol-3-0-β-D-glucoside (RG), *resveratrol-synthase* (RS), basal diet (BD), control, non-transgenic alfalfa (CA), transgenic resveratrol-glucoside accumulating alfalfa (TA).

Running title: Piceid-accumulating transgenic alfalfa and aberrant crypt foci in CF-1 mice.

Key words: resveratrol, piceid, genetically-engineered crops, aberrant crypt foci, colon cancer

Abstract

Plants have been genetically enhanced to produce a number of products for agricultural, industrial and pharmaceutical purposes. This technology could potentially be applied to providing chemoprevention strategies to the general population. Resveratrol (3,5,4' – trihydroxystilbene) is a compound that has been shown to have protective activity against a number of cancers and could be an ideal candidate for such an application. Alfalfa that was genetically modified to express *resveratrol-synthase* was used as a model in applying biotechnological approaches to cancer prevention. The transgenic alfalfa, which accumulates resveratrol as a glucoside (piceid = *trans*-resveratrol-3-*O*-β-D-glucopyranoside) (152 ± 17.5 μg piceid/g dry weight), was incorporated into a standard mouse diet at 20% of the diet by weight and fed for 5 weeks to 6-week old, female CF-1 mice (N=17-30) that were injected with a single dose of azoxymethane (5 mg/kg body weight). While the addition of resveratrol-aglycone (20 mg/kg diet) to the basal diet reduced the number of aberrant crypt foci/mouse, the transgenic alfalfa did not inhibit the number, size or multiplicity of aberrant crypt foci in the colon of the CF-1 mice relative to control alfalfa which does not accumulate resveratrol-glucoside. However, diets containing transgenic alfalfa with an exogenous β-glucosidase (860 U/kg diet) did significantly inhibit the number of aberrant crypt foci in the distal 2 cm of the colon of the mice relative to mice fed diets containing the transgenic alfalfa without the enzyme ($p<0.05$; Fisher's Combination of p-values). The β-glucosidase alone appeared to have no effect on the inhibition of aberrant crypt foci. These results suggest that piceid in transgenic piceid-accumulating alfalfa was not bioavailable.

Introduction

Colorectal cancer is the third most common cause of cancer-related illness in adults living in the United States (1). Data from case-control studies have suggested that diets rich in fruits and vegetables are protective against colon cancer (2), although findings from recent prospective studies reveal that this correlation may not indicate a causal relationship (3,4). Plants produce an array of phytochemicals as secondary metabolites for defense purposes (5). Several of these compounds have been shown to have cancer-preventing activity in laboratory studies (6). However, the concentration or bioavailability of these health-beneficial constituents is often very low in edible plants (7), which may partially explain the inconsistency between epidemiological and recent prospective studies. In recent years, there has been increased interest in developing strategies to grow crops for health-promoting purposes (8). Genetic-engineering approaches have been successfully employed to increase the yield or introduce polyphenolic compounds into plant crops (9). A successful chemoprevention strategy could involve genetically modifying crops to increase the availability of some of these bioactive constituents.

One of the most studied phytochemicals in recent years has been resveratrol (3,5,4'-trihydroxystilbene). Resveratrol is a phytoalexin synthesized in a variety of plant species in response to external stresses such as injury, UV irradiation and fungal infection (10). In the human diet, resveratrol was found in highest concentrations in red wine, grapes and peanuts (11,12). Epidemiological studies have shown an inverse correlation between the intake of red wine and the incidence of cardiovascular disease (13). It is proposed that resveratrol is partly responsible for the health benefit acquired from red wine.

Interest in resveratrol as a chemopreventive or therapeutic agent stems from an earlier report that showed that resveratrol inhibited cellular events associated with all stages of carcinogenesis - tumor initiation, promotion and progression (14). Since then, resveratrol has been shown to have growth inhibitory activity in a variety of human cancer cell lines and in animal models of carcinogenesis (15 and references therein).

A transgenic alfalfa that accumulates resveratrol (152 ± 17.5 µg resveratrol-glucoside /g dry weight) was developed to protect alfalfa against root rot (16). Alfalfa normally does not express *resveratrol-synthase* (RS) and, therefore, does not produce resveratrol. In the transgenic alfalfa, RS catalyzes the synthesis of resveratrol from the metabolic precursors p-coumaroyl CoA and malonyl CoA. Resveratrol in these plants is accumulated as *trans*-resveratrol-3-*O*-β-D-glucopyranoside (also known as piceid (RG)).

The aim of the present study was to investigate the potential effects of the alfalfa that was genetically-modified to express RS on colon carcinogenesis in the mouse model of azoxymethane (AOM)-induced carcinogenesis using aberrant crypt foci (ACF) as short-term markers. ACF are putative preneoplastic lesions that directly correlate to risk of colon cancer and tumor size in humans (17). Resveratrol was previously reported to inhibit the number and multiplicity (aberrant crypts per focus) of AOM-induced ACF in the colorectal mucosa of F344 rats (18). Mucin-depleted foci (MDF) and β-catenin accumulated crypts (BCAC) are subsets of ACF that have been identified on the bases of morphological and biochemical changes, respectively (19). Both MDF and BCAC are purported precancerous lesions but are currently not well characterized. Since ACF are widely accepted biomarkers for assessing the chemopreventive potential of agents in the colon, for our initial studies of

the transgenic resveratrol-accumulating alfalfa, we elected to use classic ACF lesions to assay the potential potency of this crop against colon cancer.

We report here that transgenic alfalfa that accumulates RG in combination with an exogenous β-glucosidase inhibited the number of ACF in the distal colon of CF-1 mice. This apparent protective effect against AOM-induced ACF was not observed with the transgenic alfalfa or the β-glucosidase alone. These results suggest that RG is not bioavailable and that the modification of polyphenolic-glucosides by endogenous enzymes may be needed to realize the potential health benefits of this transgenic crop.

Materials and Methods

Reagents and Chemicals:

Trans-resveratrol-aglycone (Rag) was purchased from the Toronto Research Institute (Ontario, Canada). The purity of the resveratrol was estimated to be > 98% by the manufacturer. α-galactosidase from *Aspirgillus niger* was purchased from the National Enzyme Company (Forsyth, MO). High-performance liquid chromatography (HPLC) grade acetone and acetonitrile were purchased from Fisher Scientific (Liberty Lane Hampton, NH). All diet ingredients were purchased from Harland Tekland (Madison, WI). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO).

Transgenic Alfalfa:

A single alfalfa (*Medicago sativa*) genotype from the Regen SY germplasm (20) was transformed with *resveratrol-synthase* (*RS*) cDNA from peanut (*Arachis hypogaena*) and analyzed for the presence and expression of RS and the accumulation of the RG metabolite at the Samuel Roberts Noble Foundation in Oklahoma as previously described (16). Clones of the transformed plant as well as untransformed control clones of the same genotype were

grown at the Samuel Roberts Noble Foundation in Oklahoma and shipped to Iowa following drying and grinding as described below. They were planted in the field at Ames, IA in adjacent plots, last harvested in October 2004. When plants reached the late bud or early flower stage, they were harvested by clipping aboveground biomass at 5 cm. Biomass was subsequently washed with water and dried in a forage drying oven at 55°C. The dried plant material was coarsely ground in a Wiley mill and the re-ground to a powder in a UDY mill with a 1mm exit filter.

Mice and Diets:

CF-1 mice (6 weeks old) were obtained from the Charles Rivers laboratory (Wilmington, MA) and housed individually in stainless steel wire-mesh cages in a temperature controlled room with a 12-hour light:dark cycle. After one week of acclimatization, the mice received one intraperitoneal injection of AOM (5 mg/kg body weight) or saline. Three days after the injections, the mice were randomized and assigned to experimental diets, which they were fed ad-libitum, for 5 weeks (N=3-10/group in 3 replicates). Mice were assigned to one of four diets: 1). A basal diet (BD) based on the standard diet recommended by the American Society for Nutritional Sciences, report for mature rats (AIN-93) (21), 2). BD with control alfalfa (CA), 3). BD with transgenic alfalfa (TA) and 4). BD with Rag.

Diets were prepared by mixing all dry ingredients with water (25% by dry weight of diet). Diets were then rolled out, cut into thin strips and dried at room temperature. Alfalfa was added at 20% by dry weight into the diets in partial replacement of dextrin (60% of dextrin added to control diets) (**Table 1**). The TA that was added to the diets contained 152 ± 17.5 µg RG /g dry weight, thus these diets contained 30.5 ± 3.5 mg RG/kg diet. *Trans*-Resveratrol-aglycone (Rag) was added to the diets in equal molar concentration (20 mg/kg

diet) to the RG by dry weight of the diet. In subsequent experiments, β-glucosidase was added to diets at 860 U/kg of dry diet. Since purified β-glucosidase was not commercially feasible at the time of this study, α-galactosidase, which was verified to have 12.5 ± 0.5 U β-glucosidase activity/mg, was used as a source of glucosidase activity. The amount of α-galactosidase (2600 α-galactosidase U/kg of diet) added to diets was determined by extrapolating the recommended human dose of Beano™ to mice on a daily energy basis (equal to 0.675 α-galactosidase U /Kcal in humans).

Resveratrol-glucoside identification in diets by HPLC analysis

To confirm the stability and uniform distribution of RG and Rag in diets, 2 g of crushed diet sample was extracted in 50 ml of a 90% methanol/10% water (v/v) solution at room temperature for 2 days under minimal light exposure. The samples were centrifuged at 1000 x g and the supernatants were evaporated and reconstituted in 1 ml methanol. The extracts were filtered and 10 µl of each extract was injected into a C18 column (2.1*150mm; Alltech Altima). Compounds were separated using a 45-minute gradient from 20-60% of acetontrile in water with a flow rate of 0.3 ml/min. Eluding peaks were monitored at λ=308 and 280 nm with a UV diode array detector (Beckman Instruments, Fullerton, CA). The spectra of peaks at 308 nm were compared to those of a *trans*-resveratrol-glucoside standard (Apin Chemicals, UK).

Analysis of ß-glucosidase activity in diets

To estimate the β-glucosidase activity of α-galactosidase, the procedures described by King (22) were followed with some modifications. Briefly, various concentrations of α-galactosidase (250-2500 ng/ml) were incubated with 1 mM of 4-methylumbelliferyl-β-D-glucoside for 30 minutes at 37°C. The reaction was stopped using sodium citrate, and the

florescence of the final product (4-methylumbeliferone) was measured using a Sequoia Turner Model 450 fluoromoter set at gain 1 with 360 nm excitation and 450 nm emission.

To measure β-glucosidase activity in the diets, 0.2 g of diet sample was crushed in a 0.1 M citric acid/0.2 M sodium phosphate buffer (pH=5.0). The suspension was vortexed and 50 µl was added to 450 µl of 1 mM 4-methylumbelliferyl-β-glucoside and incubated for 30 minutes at 37°C. The reaction was stopped by the addition of 1.5 M sodium citrate. The β-glucosidase activity of assayed samples was quantified by comparing the fluorescent readings of the final reaction to the concentration vs. the fluorescence curve of a 4-methylumbeliferone standard.

Analysis of Aberrant Crypt Foci

After feeding the experimental diets for five weeks, mice were killed by decapitation. The colon and rectum were collected from each mouse and rinsed thoroughly with phosphate buffer saline (PBS). The colon and rectums were dissected longitudinally and fixed flat in 10% buffered formalin (pH 7.5) for 24 hours. The samples were stained with 0.2% methylene blue for 10 minutes and the ACF/colon were scored for each mouse at 10-fold magnification as described by Bird (23). ACF number, size, multiplicity (number of AC/focus) and distribution were recorded in 2 cm increments, starting at the rectum in a blinded fashion. The sizes of ACF were scored with an eyepiece graticule.

Statistical Analysis

The data presented in this paper were analyzed using SAS software (SAS Institute) and are expressed as means ± SE. The first experiment was conducted in three replicates ,and the significance of the differences between ACF means, changes in body weight (final weight-initial weight) and food consumption (g/food/day) were assessed by performing a two-way

analysis of variance (ANOVA). Specific differences between groups were analyzed using a student's t-test as the post-hoc test. Because the availability of alfalfa at harvest times was limited due to the weak growth performance of both the non-transformed and transformed alfalfa lines, follow-up comparisons of diets with and without α-galactosidase were conducted in multiple studies following different harvests. In these follow-up studies, group means within experiments were analyzed using a one-way ANOVA followed by a student's t-test for specific comparisons. Treatment comparisons between follow-up experiments were made using Fisher's procedure for combining p-values (24). All statistical tests performed on the data were two-sided, and a value of $P<0.05$ was considered statistically significant.

Results

B-glucosidase activity of α-galactosidase

The relationship between the amount of 4-methylumbelliferone (4-MU) formed vs. α-galactosidase concentration was linear in the concentration range of 125-2,000 ng enzyme/ml following incubation with the substrate 4-methylumbelliferone-β-D-glucoside(4-MUG) for 30 minutes at 37°C (Figure 1). The standard curve plot for the relationship between fluorescence vs. the amount of 4-MU was linear, in the range of 0.003-0.05 µmoles of 4-MU (Figure 1, insert). This plot was used to convert fluorescent readings to 4-MU produced in the β-glucosidase-catalyzed deglycosylation of 4-MUG. Based on this assessment, the β-glucosidase activity of α-galactosidase was estimated to be 12.5 ± 0.5 β-glucosidase U (µmoles 4-MU liberated from 4-MUG) mg enzyme^{-1} min^{-1}. To test the reliability of our assay, a β-glucosidase enzyme (Sigma Aldrich, G0395 more) with a reported activity of 2.1 β-glucosidase U mg enzyme^{-1} min^{-1} was measured as well. The measured fluorescence of the β-glucosidase enzyme was linear in the concentration range of

200-4000 ng/ml (figure 2) with an estimated activity of 1.9 ± 0.03 β-glucosidase U mg enzyme^{-1} min^{-1}.

Effect of diets on body weight and food consumption

The mice gradually gained weight throughout the duration of the experiment but, as **table 2** demonstrates, the change in body weight [(final weight (29.6 ± 2.4 g) – initial weight (26.4 ± 2.1 g)] between treatment groups was not significantly (mean gain= 3.2 ± 1.6 g, p=0.5). Food intake (g/mouse/day) did not differ between treatments in the first experiment (4.0 ± 0.4 g/day/mouse, p-value=0.23). Also, there was no difference in change in body weight or in food intake in mice fed diets supplemented with α-galactosidase compared to mice that were fed diets that did not contain the enzyme in the follow-up experiments (data not shown).

Effect of treatments on formation of aberrant crypt foci in experiment 1

The number of aberrant crypts was scored for different regions of the colon as defined by 2 cm increments starting at the most distal end adjacent to the rectum. Aberrant crypts formed mainly in the distal 2 cm of the colon. The number of ACF in the most distal 2 cm of the colon was significantly lower in mice fed basal diets supplemented with Rag relative to the other diets (p-value < 0.05) (**table 2**). However, the number of ACF in the distal colon of mice fed TA was not significantly different from mice fed CA (p=0.5). Additionally, the number of ACF in the colon of mice fed CA with Rag (means= 2.5 ± 1.8 and 5.8 ± 1.5 in two replicates N= 5 and 9) was not significantly reduced compared to mice fed CA diets without Rag (means= 4.8 ± 2.5 and 8.8 ± 1.7 in two replicates N=10 each, p= 0.5 for first replicate comparison and p=0.2 for second replicate comparison).

There was no significant difference in the multiplicity (mean of all treatments= 1.4 ± 0.4 AC/focus, p=0.3) of ACF or number of ACF in the middle 2 cm (mean of all treatments=2.4 ± 2.5, p= 0.3) and proximal 1 cm (mean of all treatments=0.3 ± 0.7, p=0.1) of the colon in the CF-1 mice. There was also no significant difference in the size of ACF (mean=2.0 x 10^{-2} ± 0.7 x 10^{-2} mm^2, p=0.4), although this was assessed in only two of the three replicates. ACF were detected in 8% (4/50) of mice injected with saline and were not confined to any single treatment.

The amount of alfalfa added to the diets (20% by dry weight) was derived from previous studies in our lab showing that the number of AOM-induced ACF lesions in the colon of CF-1 mice fed diets containing resveratrol concentrations as low as 20 mg Rag/kg of diet (equivalent to the number of moles of piceid in diets containing 20% of the transgenic alfalfa) were significantly reduced (data not shown). Resveratrol at higher concentrations (40- 100 mg Rag/kg diet) significantly inhibited the number of ACF lesions formed, but the differences between these groups were not significant. Furthermore, we were concerned that supplementing alfalfa beyond 20% by dry weight would compromise the nutritional integrity of the diets.

Effect of treatments with β-glucosidase on formation of aberrant crypt foci in follow-up experiments.

As with the first experiment, the incidence of ACF was mainly limited to the distal 2 cm of the colon in all follow-up studies. The number of ACF in the entire colon and distal region of the colon was significantly reduced in the mice fed diets containing TA with α-galactosidase compared to the number of ACF in mice fed diets containing TA without exogenous α-galactosidase (**Table 3**). The difference in the number of ACF in the entire

colon or distal 2 cm of the colon was not significant between the BD and the BD containing α-galactosidase nor was the number of ACF different between diets containing 20% CA and diets containing the CA with α-galactosidase. ACF number did not differ between treatments with or without α-galactosidase in the middle or proximal region of the colon in any of these studies (data not shown). Size of ACF was assessed in only one replicate for each treatment in follow-up studies. As with the first study, there was no significant difference in ACF size (mean= $2.7 \times 10^{-2} \pm 0.6 \times 10^{-2}$ mm^2 , p=0.8) or multiplicity (mean= 1.4 ± 0.5; p=0.2) between treatments in the follow-up studies.

Discussion

The principal finding of this study was that TA that accumulates RG in combination with an exogenous β-glucosidase was effective in inhibiting the formation of ACF in the distal colon of CF-1 mice. Neither of these treatments alone was effective in reducing ACF. The number of ACF was reduced by an average of 32% and 87% in mice fed the TA with the exogenous glucosidase relative to mice fed the TA alone in two replicates where these groups were included together. ACF number was reduced by 52% in mice fed diets with purified Rag that was added in equal molar concentrations to that of the RG in TA. The protective effect of the TA in combination with β-glucosidase was not due to the presence of other plant metabolites present in alfalfa since CA supplemented at 20% by dry weight into the diet did not inhibit ACF development in the colon of CF-1 mice regardless of whether or not β-glucosidase was added. These results imply that RG may not be bioavailable and modification of secondary metabolites by endogenous enzymes in the plant is an important consideration when assessing the potential health-benefits of a crop genetically-enhanced to produce high levels of these compounds. To our knowledge this is the first *in-vivo*

demonstration of the potential health benefits of a genetically modified crop against a biomarker of cancer.

Resveratrol is found in low and variable amounts in relatively few sources in the human diet. Resveratrol content is very low in peanuts (< 1μg/g wt) and grapes (< 10 μg/g fresh wt), which are two major dietary sources of resveratrol (12, 25). Furthermore, the majority of resveratrol found in grapes is in the form of *cis*- or *trans*-piceid with the aglycone comprising only a small fraction of the detectible levels of resveratrol (< 0.6 μg Rag/g fresh wt). In the human diet, resveratrol is found in highest concentrations in red wine. However, analysis of different red wines revealed that *trans*-resveratrol-aglycone content of wines can vary (0.1 – 14 mg/L) depending upon the cultivar of grape used, the climate in which the grapes were grown, and the maceration process followed (12, 26, 27, 28). *Trans*-piceid has also been identified at appreciable levels in red wine, in some cases as high as 50.8 mg/L. Genetic-engineering strategies could provide a means to increase the dietary availability of resveratrol to humans through additional food options.

In addition to alfalfa, several other plants have been genetically modified to express *resveratrol-synthase* for agronomical purposes, including tobacco (29), rice (30), barley (31), kiwi (32), and apple (33). Resveratrol was reported to accumulate as a glucoside in all of these plants when the presence of the resveratrol was assessed.

The present study suggested that RG was not as bioavailable or as bioactive as Rag for ACF prevention, since the addition of an exogenous β-glucosidase to TA was required to achieve a reduction in AOM-induced ACF in the colorectal mucosa of CF-1 mice. TA alone was not protective against AOM-induced ACF. We hypothesize that the supplemented glucosidase catalyzed the liberation of the more bioavailable aglycone moiety from RG in

diets containing the TA. Diets which contained the exogenous glucosidase with CA did not reduce the number of ACF in the colon of CF-1 mice, suggesting that the protective effect of the glucosidase was associated with the RG in the transgenic alfalfa. This was a surprising observation as alfalfa contains numerous secondary metabolites (34, 35). The total concentration of flavonoids in alfalfa has been shown to range from 0.24 to 0.78% in dry matter with the majority of identified compounds being glycosides of apigenin, luteolin and tricin (34). The TA used in our study contained, on average, 0.015% RG by dry weight. It is possible that most of the natural-occurring polyphenols in alfalfa are not bioactive or some of the more active compounds are not present in very high concentrations.

Few studies have reported on the bioactivity or bioavailability of RG. RG was shown to inhibit platelet aggregation (36) and inhibit thymine incorporation into DNA in Lewis lung carcinoma cells (37). However, the effective dose in the later study was 100 μM (IC > 1000 μM), bringing into question the potential bioactivity of RG. In the Kimura et al study (37), RG inhibited tumor growth in the paw of C57BL/6 mice that were transplanted with Lewis lung carcinoma cells, although his protective activity was achieved by the administration of very high doses of RG at 300 mg kg^{-1} daily for 32 days. Lower doses were not reported in this study. Assays conducted in Caco-2 monolayer systems reveal that the sodium-glucose co-transporter (38) and lactase phorizin hydrolase (39) may play an important role in the absorption and deglycosylation of RG in the intestine. In the former study, the uptake of Rag in Caco-2 cells was more rapid than RG with the intercellular concentrations of Rag remaining at least 4 times higher than RG at all time points examined in Caco-2 cells that were treated with 150 μM of each compound (38). In a recent study, RG was detected in a variety of tissues, including the brain, heart, liver and lungs in Wistar rats within 20 minutes

of the rats receiving a single, oral dose of piceid (50 mg/kg body weight) suspended in a 5% sodium carboxymethylcellulose solution (40). Plasma concentrations of RG reached a peak concentration of 0.9 ± 0.4 μM at 20 minutes post-dose administration. Unfortunately, side-by-side comparisons with Rag where not featured in this study. To our knowledge, the colon bioavailability of piceid has not yet been examined.

Rag was shown to inhibit chemically induced ACF in rodents in at least two other studies. Rag at daily doses of 200 μg kg^{-1} body weight via 10% ethanol in drinking water reduced the number, multiplicity and size of AOM-induced ACF in the colorectal mucosa of F344 rats (18). In a more recent study, Sengottuvelan et al showed that Rag (8 mg kg^{-1} body weight p.o., daily) reduced the number, multiplicity and size of ACF in rats injected weekly with 1,2-dimethylhydrazine (DMH) (20 mg kg^{-1} body weight for 15 weeks) (41). This group has also reported that Rag reduced the number of DMH-induced colonic tumors in rats regardless of whether Rag was administered before or following DMH injections (41, 42). In contrast, reports of resveratrol treatment on tumor incidence in C57BL/6J APC^{Min} mice with genetic colon cancers caused by mutant adenomotosis polyposis coli (APC) have been inconsistent (43, 44). Studies with Rag have been conducted in Min mice between the ages of 4-5 weeks, which may represent a more advanced stage of cancer since preneoplastic lesions begin to develop in the small intestine of these mice *in utero* (45) and may indicate that resveratrol may be more valueable as a preventive rather than a therapeutic agent in the colon. A number of biological activities have been ascribed to resveratrol which may, at least partially, explain any anti-cancer properties that the chemical may possess. Resveratrol has been demonstrated to prevent free-radical formation and have anti-mutagenic activity; inhibit activity of cytochrome P450 enzymes; inhibit cyclooxygenase-2 catalyzed reactions;

induce phase-2 drug metabolism; induce cell cycle arrest and apoptosis (46 and references therein). Additional studies are warranted to determine resveratrol's mechanism of action in the colon.

In conclusion, we have demonstrated that transgenic alfalfa that accumulates resveratrol-glucoside did not inhibit AOM-induced ACF in the colorectal mucosa of CF-1 mice, but the addition of an exogenous glucosidase enzyme to diet preparations containing the transgenic alfalfa seemed to be protective against AOM induction of ACF in the distal colon of CF-1 mice. Our data suggests that future studies on the bioavailability and bioactivity of RG are essential in determining the therapeutic value of resveratrol as a nutraceutical. In addition, we have offered a strategy whereby other transgenic crops may be analyzed for their heath-benefiting value.

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Table 1: Comparison of Diets

	Basal	Diets with alfalfa
Casein (g/kg)	200	200
Dextrin (g/kg)	499.5	299.5
Alfalfa (g/kg)	-	200
Dextrose (g/kg)	150	150
Fiber (g/kg)	50	50
Mineral mix (g/kg)	35	35
Choline (g/kg)	2.5	2.5
Methionine (g/kg)	3.0	3.0
Vitamin mix (g/kg)	10	10
Corn oil (g/kg)	50	50

Trans-resveratrol-aglycone was added to some basal and control alfalfa diets at 0.002% by dry weight which was equivalent to the number of moles of piceid present in diets containing the transgenic alfalfa which accumulates 170 µg piceid/g wt of plant. In follow-up studies, α-galactosidase was added at 860 β-glucosidase U (2600 α-galactosidase U/kg) to basal, control alfalfa and transgenic alfalfa diets.

Table 2: Number of azoxymethane-induced Aberrant Crypt Foci and multiplicity (Average number of aberrant crypts per focus) in the colon of CF- mice.

Treatment	N	Gain in Body Weight (Initial Body weight) (g) (ANOVA p-value=0.5)	Total ACF/mouse (ANOVA p-value =0.09)	Region of colon (ACF/mouse)			Multiplicity (AC/focus) (ANOVA p-value=0.3)
				D (ANOVA p-value=.0023)	M (ANOVA p-value =0.3)	P (ANOVA p-value =.11)	
Basal (BD)	25	2.8 ± 0.3 (26.9 ± 0.4)	8.4 ± 1.1	5.4 ± 0.7	2.8 ± 0.5	0.2 ± 0.2	1.5 ± 0.1
Control Alfalfa (CA)	17	3.3 ± 0.5 (26.3 ± 0.5)	7.9 ± 1.3	4.3 ± 0.9	3.1 ± 0.6	0.5 ± 0.2	1.5 ± 0.1
Transgenic Alfalfa (TA)	21	3.1 ± 0.4 (27.1 ± 0.5)	7.2 ± 1.2	5.1 ± 0.8	1.9 ± 0.6	0.4 ± 0.2	1.5 ± 0.1
BD + resveratrol-aglycone	30	3.4 ± 0.3 (25.8 ± 0.4)	4.4 ± 1.0[1]	1.9 ± 0.6[2]	2.2 ± 0.5	0.3 ± 0.1	1.3 ± 0.1

Values represent mean ± SE (2-way ANOVA with student's t-test post-hoc analysis; SAS software). This experiment was conducted in three replicates with between 3 and 10 mice per group. The regions of the colon are defined as follows: D= distal 2 cm, M= second 2cm from distal end, P=proximal 1 cm. [1] $p<0.05$ compared to BD and CA groups for total ACF. [2]$p<0.05$ compared

to BD, CA and TA treatments in the rectal region of the colon. ACF size was also assessed in some studies but no significant difference between treatments was observed in all of the studies where this parameter was examined ($p>0.33$ for all studies; one-way ANOVA with a student's t-test as the post-hoc analysis; SAS software). Mice from each treatment were also injected with saline. Aberrant crypts were observed in 8% of saline treated mice (4/50), but were not confined to any single treatment.

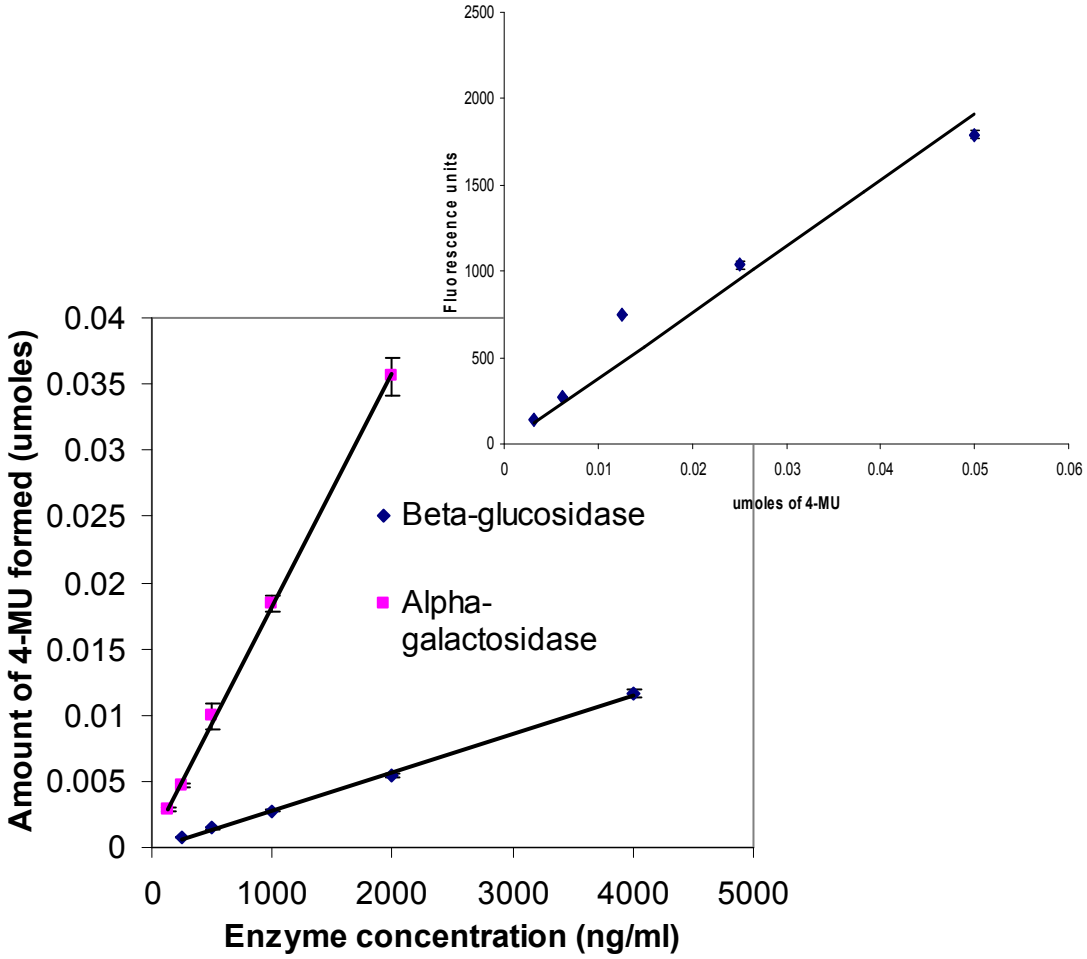
Table 3: The addition of β-glucosidase activity to Transgenic alfalfa diets reduced the number of ACF in the colon of CF- mice.

Treatment Comparison	Study	Without Enzyme			With Enzyme			p-value* (Total colon)	p-value* (Distal 2 cm)
		N	Mean ± SE (Total colon)	Mean ± SE (Distal 2 cm)	N	Mean ± SE (Total colon)	Mean ± SE (Distal 2 cm)		
			BD	**BD**		**BD+E**	**BD+E**		
BD vs. BD+E	1	10	6.9 ± 1.5	3.8 ± 0.9	10	9.9 ± 1.5	4.6 ± 0.9	> 0.1 (0.2, 0.1)	0.7 (0.5, 0.6)
	2	10	8.6 ± 1.3	5.9 ± 0.1	9	11.7 ± 1.4	6.6 ± 1.0		
			CA	**CA**		**CA+E**	**CA+E**		
CA vs. CA+E	1	10	3.3 ± 1.5	1.5±0.93	10	5.1 ± 1.5	2.2 ± 0.93	>0.3 (0.3, 0.5)	>0.5 (0.6, 0.4)
	2	4	10.3 ± 2.4	7.3 ± 1.6	4	8.0 ± 2.1	5.3±1.6		
			TA	**TA**		**TA+E**	**TA+E**		
TA vs. TA+ E	1	10	3.0 ± 0.9	1.9±0.8	10	0.8 ± 0.9	0.6 ± 0.4	< 0.01 (0.06, <0.01)	<0.01 (0.1, <0.005)
	2	3	10.4 ± 2.4	8.7 ± 1.8	3	1.3 ± 2.4	1.0±1.8		

Values represent mean ± SE. Diets are defined as follows: BD= basal diet, CA=control alfalfa, TA=transgenic alfalfa, E= α-galactosidase and R= resveratrol-aglycone. Diets with α-galactosidase contained 2600 α-galactosidase U/kg diet and 860 β-glucosidase U/kg diet. *Comparing no enzyme vs. enzyme treatments p-values were derived using Fisher's combination of tests of significance analysis. P-values for individual studies are listed in the parantheses under the combined p-values for each comparison.

Figure 1: β-glucosidase activity of α-galactosidase (National Enzyme Company) and β-glucosidase (G0395, Sigma Aldrich). B-glucosidase activity was expressed as a function of 4-methylumbelliferone formed after incubating the enzymes with the substrate 4-methylumbelliferone- β-glucoside for 30 minutes @ 37ºC. Values represent means ± SE (N=6). Linear parameters were as follows: (for α-galactosidase) slope= 2 x 10^{-5}, y-intercept= 0.0007 and R^2=0.9993; (for β-glucosidase) slope=3 X 10^{-6}, y-intercept=--.0001 and R^2=0.997. Fluorescent units (recorded at 360 nm excitation and 450 nm emission) were converted into µmoles of 4-methylumbelliferone formed based on the relationship between fluorescence and concentration of 4-MU authentic standards (see insert). This relationship was linear in the concentration range of 0.003-0.05 umoles of 4-MU (linear parameters were: slope= 34142, y-intercept = 136.89 and R^2= 0.9683).

Figure 1:



CHAPTER 3: THE IMPORTANCE OF HYDROLYSIS OF PICEID TO RESVERATROL-AGLYCONE FOR INHIBITION OF AZOXYMETHANE-INDUCED ABERRANT CRYPT FOCI IN CF-MICE FED PICEID ACCUMULATING TRANSGENIC ALFALFA

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Abbreviations: Aberrant crypt foci (ACF), trans-resveratrol-aglycone (Rag), Piceid = trans-resveratrol-3-O-β-D-glucoside (RG), basal diet (BD), control, non-trasnsgneic alfalfa (CA), transgenic resveratrol-glucoside accumulating alfalfa (TA), lactase-phlorizin hydrolase (LPH), azoxymethane (AOM)

Abstract

Based on our previous findings, we tested the hypothesis that piceid (resveratrol-glucoside, RG) is not bioavailable from transgenic alfalfa that accumulates RG. Transgenic alfalfa (TA) which contained 193 ± 18 μg RG /g dry weight was incorporated into diets at 20% by dry weight and fed to female, 6-week old CF-1 mice for 5 weeks. The supplementation of 860 β-glucosidase U/kg diet to transgenic alfalfa and control diets with authentic RG reduced the number of azoxymethane (AOM)-induced aberrant crypt foci (ACF) in the colon of CF-1 mice fed these diets by 36% and 41%, respectively, compared to transgenic alfalfa and RG, alone. The incidence of Rag detection was significantly less in the TA group relative to TA with E1 (60 vs. 100%, respectively, $p<0.05$). Colonic concentrations of resveratrol-aglycone (Rag) (< 0.5 nmol/g tissue) in mice fed TA with enzyme (0.22 ± 0.18 nmol/g tissue) tended to be higher than in animals fed diets without the enzyme supplement (0.1 ± 0.08 nmol/g tissue) ($p= 0.09$). Rag could be detected in the colonic, small intestinal epithelial and plasma 1, 3 and 7 hours following intrgastric feeding of 25 mg resveratrol-aglycone/kg body weight. However, neither RG or Rag could be detected in these tissues following the same time-course in mice intragastrically fed 25 mg RG/kg body weight. The use of *N*-(*n*-butyl)-deoxygalactonojirimycin (NB-DGJ), an inhibitor of lactase-phlorizin hydrolase (LPH), in transport studies with everted jejunal sacs from CF-1 mice (N=8) suggested that LPH is involved in the intestinal deglycosylation of RG. However, the concentration of Rag measured in intestinal mucosa of preparations incubated in 100 μM Rag was much greater than the amount of piceid and resveratrol-aglycone detected in the mucosa of intestinal sacs incubated in equal molar concentrations of RG. Collectively, our

studies suggest that the intestinal bioavailability of RG is much less than that of the Rag and that bioavailability was related to inhibition of AOM-induced ACF.

Introduction

In recent years, genetically-engineering strategies have been employed to produce plants with higher yields of constituents that may be beneficial to human health (Grusak MA and DellaPenna, 1999; Yan and Kerr, 2002; Bouis et al, 2003; Celec et al, 2005). The new products include plants with high concentrations of flavanoid phytochemicals (Muir et al, 2001; Bovy et al, 2002; Schijlen et al, 2006; Sreevdya et al, 2006). Many of these compounds have been individually studied for their anti-oxidant and anti-inflammatory properties and may potentially offer a variety of health benefits (Prior, 2006). However, the efficacy of GM products designed to improve human health through increased yields of these plant metabolites are not well documented.

Resveratrol (*trans*-trihydroxystilbene) is a polyphenolic compound found in the diet in highest concentrations in red grapes, red wine and berries and in certain herbs such as *Polygonum caspidatum* (Ribeiro de Lima et al, 1999; Wang et al, 2002; Burns et al, 2002). Resveratrol is one of the constituents of red wine that has been purported to have protective activities against cardiovascular disease (reviewed by Demlas et al, 2005). In addition to cardiovascular disease, Rag may be protective against other diseases and conditions associated with oxidative damage such as neurodegenerative disorders (Han et al, 2006), inflammation (Martin et al, 2006) and cancer (Jiang et al, 1997). Rag was shown to inhibit cell growth and induce cell cycle arrest in SW480, HT29 (Liang et al, 2003) and Caco-2 (Wolter et al, 2001) human colon cell lines and inhibit the formation of axoxymethane

(AOM)-induced aberrant crypt foci (ACF) (Tessitore et, 2000) and 1,-2-dimethylhydrazine-induced tumors in rodents (Sengottuvelan et al, 2005). The later animal experiments indicate that Rag may be bioavailable to the colon.

Interest in applying recombinant DNA strategies to enrich new plant species with resveratrol has surfaced in recent years due to the purported phytoalexin activity and health benefits of resveratrol. Thus, resveratrol-rich crops may have both agronomical and consumer-oriented benefits. To date, *stilbene synthase* has been ectopically expressed in rice (Stark Lorenzen et al, 1997); barley and wheat (Leckland et al, 1998), tomatoes (Thomzik, 1997), kiwi (Kobayashi et al, 2000), apple and arabidopsis (Yu et al, 2006). Resveratrol accumulated as *trans*- or *cis*-piceid (resveratrol-glucoside) (RG) in all of these plants where the presence of resveratrol was assessed. Likewise, resveratrol is found predominantly as a glucoside in natural sources (Baur and Sinclair, 2006). Interestingly, the bioactivity of resveratrol has largely been demonstrated with Rag, while the glucoside forms of resveratrol have received limited attention.

We were interested in examining the colon chemopreventive properties of transgenic alfalfa (TA) which accumulated RG (Hipskind and Paiva, 2000). Previously, we reported that the TA inhibited the formation of AOM-induced ACF in the colon of CF-1 mice only when diets containing the alfalfa were supplemented with an exogenous β-glucosidase (Kineman et al, submitted). Our findings suggested that RG may not be bioavailable and liberation of the Rag via deglycosylation may be required before the potential benefits of resveratrol are realized from RG-rich sources.

Studies suggest that the intestinal absorption of polyphenols is dependant on the release of aglycones from glucoside conjugates by intestinal or microflora β-glucosidases (Nemeth et

al, 2003). Lactase Phlorizin hydrolase (LPH) is an extra-cellular enzyme that has β-glucosidases activity and is located at the brush boarder of the intestinal epithelium. The lactase catalytic site of LPH has been shown to play an important role in the deglycosylation of some polyphenolic-glucosides, including quercetin-3' and quercetin-4'-glucoside (Day et al, 2000 and Day et al, 2003), and other glucosides such as pyridoxine-5-β-D-glucoside (Mackey et al, 2002). Recently, a role for LPH in the intestinal cleavage of RG to resveratrol-aglycone was supported in Caco-2 cells (Henry-Vitrac et al, 2006).

The objective of this study was to explore a tactic to increase the anti-cancer activity of the TA through the addition of purified β-glucosidase and to determine if bioavailability of RG with these diet preparations may explain the improved inhibition the formation of AOM-induced ACF in the colon mucosa of CF-1 mice. We tested the hypothesis that RG is not bioavailable by measuring Rag and RG levels in the colon and plasma of CF-1 mice following administration of diet preparations containing the TA with and without the supplemented β-glucosidase. Furthermore, we assessed the potential substrate suitability of RG for LPH, an endogenous glucosidase of the small intestine in a mouse everted-jejunal sac model. Our findings indicate that RG is a substrate for intestinal LPH, but that its colonic bioavailability may be much less than that of Rag.

Materials and Methods

Plant material and greenhouse conditions

A single alfalfa (*Medicago sativa* cv. Cimarron) was transformed with *resveratrol-synthase* (*RS*) cDNA from peanut (*Arachis hypogaena*) at Samuel Roberts Noble Foundation in Oklahoma as previously described for cv. Regen SY (Hipskand and Paiva, 2000).

Cuttings from the transformed plants were shipped to Iowa State University where they were grown in a greenhouse at 25°C with a 16 hour light cycle. Transformed plants were crossed to alfalfa cultivar Pioneer 5246 and seeds from the crossed pods were collected and grown in the greenhouse. Plants were propagated through stem cutting by planting stem internodes that were trimmed free of mature leaves. Rooted cuttings were grown in 3.5 x 3.5 inch plots under greenhouse conditions.

PCR analysis of Transgenic alfalfa lines

Genomic DNA was extracted from 0.1 g of alfalfa green leaf tissue as previously described (Doyle and Doyle, 1989). To identify plant crosses, plants were screened for the kanamycin resistance gene (NPTII) that was included on the binary vector used in the transformation of the plants. The *resveratrol-synthase* (*RS*) gene was not directly assessed, since *RS* shares 70-80% homology with *chalcone synthase* at the DNA level (Tropf et al, 1994).

Specific NPTII primers (5' TCACTGAAGCGGGAAGGGACT 3') and (5' CATCGCCATGGGTCACGA 3') were used for PCR. For the reaction, one microgram of genomic DNA was combined with 0.8 µM primers (final concentration), 0.2 µM dNTPs, x U of Taq polymerase (Promega, Madison, WI) in 1 X PCR buffer (Promega, Madison, WI) adjusted to a final volume of 25 ul. Thermocycler (Biorad, Hercules, CA) conditions were as follows: denature at 94° C for 2 min, anneal at 63°C for 1 min, elongate at 72°C for 1 min for a total of 30 reactions. Twenty-five µl of PCR product was separated by agarose gel electrophoresis and visualized by ethidium-bromide staining under UV light.

Identification of resveratrol-glucoside in plants

Stems and leaves from alfalfa were harvested, dried at 55°C in a forage drying oven and ground to a fine powder using a Wiley Laboratory mill with a 1 mm screen (Thomas Scientific, Swedesboro, NJ). To analyze dry material for RG accumulation, the procedures described by Hipskind et al (2002) were followed, with some modifications. RG was extracted from 2 g of powdered plant material in 90% methanol/10% water overnight at room temperature. Samples were centrifuged at 4000 x g and 10 ml of supernatant was dried using a rotary evaporator. The extract was then dissolved in 500 ul methanol, filtered using syringe filters (0.25 µm PTFE, Altech) and 10 ul of samples was analyzed using reverse-phase high-performance liquid chromatography (HPLC). A Beckman System Gold HPLC was used equipped with a 168 diode array detector and an Alltech Alltima 2.1 x 150 mm C18 column and Alltima C18 precolumn. The mobile phase consisted of milliQue (MQ) water (A) and 100% HPLC grade acetonitrile (Fisher) (B). Separation was carried out using a 45 minute linear gradient from 10-60% B at a flow rate of 0.3 ml/min. The column was equilibrated with 100% B 10 minutes prior to each analysis. UV spectra were recorded at 308 nm (optimal for *trans*-RG and *trans*-Rag) and 280 nm (optimal for *cis*-RG and *cis*-Rag). The identification of RG and Rag in samples was confirmed by comparing UV spectra at 308 nm to standards of both compounds. The recovery of a spiked RG standard was 99.8% (N=5).

Diets

The control diet consisted of the standard diet recommended by the American Society for Nutritional Sciences report for mature rats (AIN-93) (Reeves et al, 1993). Diets were prepared by mixing all dry ingredients with water (25% by dry weight of diet) into a slurry. Diets were then rolled out, cut into thin strips and dried at room temperature for two days.

Alfalfa was added at 20% by dry weight into the diets in partial replacement of dextrin (60% of dextrin added to control diets) as described previously (Kineman et al, submitted). The TA that was added to the diets contained 193± 18 µg RG /g dry weight (100 µmol RG/kg diet). *Trans*-Rag (Sigma-Aldrich, St. Louis, MO) and *trans*-RG (trans-resveratrol-3-glucoside) (Apin Chemicals, UK) weere added to the diets at a concentration of 100 µmols/kg diet, which was equivalent to the amount of RG measured in the transgenic alfalfa diets. α-galactosidase (E1) from almond (National Enzyme Company, Forsyth, MO) and ß – glucosidase (E2) (G4511, Sigma Aldrich, St. Louis, MO) from almond were added to diets at 860 U/kg of diet (Kineman et al, submitted). Enzymes were added along with the water into the diets and mixed with the other ingredients. Diets were stored at -20°C and administered every two days. The stability and uniform distribution of RG and Rag in the diets during the 5-week duration of the studies was confirmed by HPLC analysis. The stability of β-glucosidase activity in the diet over the 5-week period was confirmed by a methylumbelliferone β-D-glucoside assay as previously described (Kineman et al, submitted). Additionally, the stability of β-glucosidase activity in the diet was determined at a pH of 3, 5 and 7, to mimic the conditions of the stomach (pH=3) and small intestine (pH=7).

Animals

CF-1 mice (6 weeks old) were obtained from the Charles Rivers laboratory (Wilmington, MA) and housed individually in stainless steel wire-mesh cages in a temperature controlled room with a 12-hour light:dark cycle. Mice were fed control (basal) diet, ad-libitum, upon arrival. Body weights were recorded weekly and food consumption was monitored every two days.

Animal experiments:

1). Aberrant Crypt foci (ACF) Study

To induce ACF, the mice were intraperintonially injected with a single dose of azoxymethane (5 mg/kg body weight) (Midwest Research Institute, Kansas City, MO) or 0.1% saline. Three days after the injections, the mice were randomized and assigned to one of the following 12 experimental diets: 1) control diet, 2) control diet with RG, 3) control diet with Rag, 4) control diet with non-transgenic alfalfa (CA), 5) control diet with TA, 6-10) diets 1-5 supplemented with β-glucosidase enzyme (E2) and 11-12) diets 4 and 5 supplemented with an alpha-galactosidase enzyme (E1). The mice were fed these diets ad-libitum for 5 weeks.

Following the five week experimental period, mice were killed by decapitation. Whole trunk blood was collected into heparin-coated tubes and immediately placed on ice and shielded from light exposure. Colons were excised from the mice and rinsed thoroughly with ice-cold 0.1 % saline to remove luminal contents. The colons were then dissected longitudinally and fixed flat in 10% buffered formalin (pH 7.5) for 24 hours. The samples were stained with 0.2% methylene blue (Sigma Aldrich, St. Louis, MO) in 0.1% saline for 10 minutes and the ACF/colon were scored for each mouse at 10 fold magnification using the criteria defined by Bird (1995). ACF number, size, multiplicity (number of AC/focus) and distribution were recorded in 2 cm increments, starting at the rectum in a blinded fashion. Sizes of ACF were scored with an eyepiece graticule.

2). Colon and plasma concentrations of piceid and resveratrol-aglycone in mice fed diets from ACF study

CF-1 mice were fed the following diets for five weeks: 1) control diet, 2) control diet with Rag, 2) control alfalfa (CA), 3) CA with E1, 4) transgenic alfalfa (TA) and 5) TA with E1. All food was removed from cages 4 hours prior to killing all the mice by decapitation. Whole trunk blood was collected into heparin-coated tubes and immediately placed on ice. Colons were excised from the mice and rinsed thoroughly with ice-cold saline and snap frozen in liquid nitrogen.

Analysis of plasma and tissue samples for resveratrol

Plasma was isolated from whole blood by centrifugation at 500 x g for 15 minutes. Aliquots (50 µl) of plasma containing 20 uM flavone (2-phenyl-4H-1-benzopyran-4-one) (Sigma Aldrich, St. Louis, MO) as an internal standard were placed in Eppendorf tubes for enzyme digestion with 980 U β-glucuronidase containing 9.1 U sulfatase (β-glucuronidase from Helix pomitia, Sigma Aldrich, G-0876) for 18 hours at 37°C. The reaction was terminated with an equal volume of 100% methanol. The samples were centrifuged at 10,000 x g for 15 minutes and the supernatants were filtered.

Colons were weighed and then homogenized in 2 ml of 100% methanol using a Polytron homogenizer for 40 s. Flavone was added as an internal standard to each sample (20 uM final concentration). The samples were centrifuged at 4000 x g for 15 minutes. The supernatants were dried under nitrogen gas, reconstituted in 500 ul methanol and filtered prior to LC-MS analysis. Colon extracts were not digested with β-glucuronidase/sulfatase prior to analysis, since results described below indicated that there was no difference in the amount of Rag detected in the colonic epithelial between digested and undigested samples.

LC-MS analysis of plasma and colon extracts

In order to confirm the identity and amount of Rag and RG in the plasma and colon extracts, LC-MS analysis was carried out using a 1100 Series resverse-phase HPLC (Agilent Technologies) coupled to a mass selection trap SL detector (Agilent Technologies) equipped with a Turbo ionspray source with the capillary voltage set at 0.7 and heated to 350°C. Acquisition was performed in negative ion mode using the deprotonated molecule of trans-Rag (m/z 227) and RG (m/z 389) as precursor ions. Compounds were separated using an Alltech Alltima 2.1 x 150 mm C18 column and Alltima C18 precolumn C18 column (Alltech). The solvent system consisted of a 16 minute linear gradient from 10-60% acetonitrile in 0.1% formic acid. At the end of the gradient the column was flushed with 100% acetonitrile for 2 minutes. Eluted peaks were monitored at 308 nm and 280 nm prior to being introduced into the ionization chamber. Quantitative determination of Rag was based on an external standard. A calibration curve was conducted in the range of 30-4000 pmol and was linear over the entire range with correlation coefficient value of 0.9973 (figure 1). The recovery of the flavone internal standard was 94.7 ± 2%.

3). Plasma, colonic and small intestinal epithelium concentrations of RG and Rag in mice fed high concentrations of both compounds

The colonic epithelium was measured for Rag and RG concentrations following the administration of high doses of each compound to mice, to determine the relative amounts of these compounds that can reach this tissue compared to the small intestinal epithelium and plasma, since this is the precise site where ACF form. Six-week old CF-1 were fed control diets for a one-week wash-out period. All food was removed from the cages 12 hours prior to administration of RG or Rag. Mice were intragastrically fed 25 mg/kg Rag or RG in 10% ethanol via gavage and killed 1, 3 or 7 hours post-dose administration by decapitation.

Colons were excised and luminal contents were flushed with 0.1 % saline. Trunk blood was collected into heparin-coated tubes and immediately placed in ice.

Colonic and small intestinal epithelial cells were isolated using the procedures described by Brasitus and Kereztes (1983) with minor modifications. Colons were rinsed with solution containing 0.015 M NaCl and 0.001 M dithiothreitol (DTT) and tied at one end. The colon sacs were filled with 10% fetal calf serum in 1.6% Joklik's modified minimal essential medium, tied shut at the other end, and incubated @ 37°C for 12 minutes in 0.1% in a 25 ml flask. Following the incubation period, the colons were removed from the water bath and the inner media drained. The colonic sacs were then filled with Weiser B solution (0.0015 M EDTA and 0.0005 M DTT in 0.1 M phosphate-buffer, pH=7.2) and incubated at 37° C for 15 minutes while shaking. The Weiser B contents were collected into 15 ml centrifuge tubes. Flavone was added to each sample as an internal standard (final concentration = 20 uM) and the samples were homogenized for 30 seconds. Methanol (4 X volume) was added to each sample and the samples were vortexed for 1 minute each and centrifuged at 4000 rpm for 15 minutes. Supernatants were collected and methanol was evaporated off under nitrogen gas and redissolved in 500 µl potassium phosphate buffer (5 mM, pH 6.7). Subsamples were digested with 980 U β-glucuronidase and 9.1 U sulfatase (β-glucuronidase from Helix pomitia, Sigma Aldrich, G-0876) at 37°C for 24 hours. Resveratrol was extracted from aqueous solution with equal volumes of ethyl acetate (3 x). The ethyl acetate fractions were pooled and dried under nitrogen gas. The extracts were reconstituted in 500 µl methanol and filtered prior to reverse-phase HPLC analysis as described above. One-hundred µl of each plasma sample was processed and analyzed by HPLC as described above. The calibration curves of RG and Rag were made by injecting methanolic solutions

of each in a concentration range of 0.5 to 25 µmol/L, which were linear with a correlation coefficient value of 0.9634 and 0.9965, respectfully. The detection limits of RG and Rag were determined to be 0.02 µmol/L for each compound. The recovery of the flavone internal standard was 72 ± 2% for the colon and small intestinal extracts and 92 ± 5% for the plasma extracts. The relative standard deviation of 5 concentrations over 5 different times was 1.6-2.5 for the RG and 1.8-3.9 for Rag.

4). Role of lactase-phlorizine hydrolase in intestinal deglycosylation of piceid

The substrate suitability of RG for the lactase catalytic site of LPH was examined in the jejunum of CF-1 mice, since the highest activity of LPH in humans is found in this section of the small intestine (Skovbjerg et al, 1981). For these studies, everted, jejunal sacs from CF-1 mice were incubated with piceid in the presence or absence of *N*-(*n*-butyl)-deoxygalactonojirimycin, a specific inhibitor of lactase (Andersson et al, 2000).

Optimization of lactase inhibitor concentration

To indirectly assay the small intestines of CF-1 mice for lactase activity, the procedure outlined by Day et al (2003) were followed. Briefly, mice were killed by cervical dislocation prior to removal of their small intestine via abdominal incision. The jejunum was identified in each mouse, excised and rinsed with Krebs-Henselect bicarbonate buffer (115 mM NaCl, 2.5 mM KCl, 2.5 mM $MgSO_4$, 1.9 mM $CaCl_2$ x $2H_2O$, 5.6 mM glucose, 1.4 mM NaH_2PO_4 x $2H_2O$ and 25 mM $NaHCO_3$ at pH=7.4). Approximately 2 cm sections were cut from the proximal jejunum, weighed and everted. One end of the everted intestine was ligatured and the intestine was filled with Krebs bicarbonate buffer using a bulb end needle attached to a 1 ml disposable syringe at the other end of the intestine. The needle was removed and the intestine was ligatured to form a sac. The everted intestinal sacs were suspended in organ

baths at 37°C in 25 ml Erlenmeyer flasks containing incubation media (10 mls) as described below and continuously gassed with 95% O_2/CO_2.

Pre-weighed everted, intestinal sacs from CF-1 mice were incubated for 15 minutes @ 37ºC in various concentrations (0-500 uM) of *N*-(*n*-butyl)-deoxygalactonojirimycin (NB-DGJ) (EMB Biosciences, San Diego, CA) along with ^{14}C-lactose (final concentration = 4.63 kBq/mL) (GE Health Sciences) in Krebs bicarbonate buffer that was continuously gassed with 95% O_2/ 5%CO_2. The sacs were removed from the organic baths and the outer mucosa of the sacs was rinsed with Krebs bicarbonate buffer and blotted with filter paper to remove excess solution. The inner serosal solutions were drained into 20 ml glass scintillation vials with 9.5 mls of Hionic-fluor™ scintillation cocktail (PerkinElmer, Wellerley, MA). The tissues were placed in 20 ml glass vial and digested in 2 mls of Solvable™ tissue solubilizer (PerkinElmer) for 2 hours at 55°C. Hionic-fluor™ scintillation cocktail (8 mls) was added to the digested samples. The ^{14}C isotope was measured in the tissue extracts using a 1900 TR TriCarb Packard Liquid Scintillation Analyzer.

The amount of ^{14}C-glucose cleaved from the ^{14}C-lactose and absorbed into the intestinal sacs was determined as a function of the measured radioactivity of the serosal solutions + tissue normalized to tissue weights.

Transport studies using lactase inhibitor

Everted, jejunal sacs were incubated with piceid in the presence and absence of NB-DGJ to determine if RG is a substrate for LPH. Preweighed, everted intestinal sacs from CF-1mice were incubated in 100 uM RG or 100 µM Rag in the presence or absence of 300 uM NB-DGJ in Krebs bicarbonate buffer as described above. The intestinal sacs were removed from the water bath following incubation at 37°C for 15 minutes and blotted once on filter

paper. The inner serosal solution was collected into 1.5 ml microcentrifuge tubes and vortexed with an equal volume of 1 mM ascorbic acid in methanol containing 20 uM flavone (Sigma-Aldrich) as an internal standard. The extracts were centrifuged at 16,000 x g and the supernatants were dried under vacuum using a rotary evaporator. The extracts were reconstituted in 500 ml methanol and filtered using a syringe filter prior to HPLC analysis.

The mucosa was scrapped from the intestinal sacs using two microscope slides. The mucosal collections were then transferred to 2 ml microcentrifuge tubes that contained 2 mls 1 mM ascorbic acid in methanol along with 20 uM flavone. The mucosal samples were homogenized for 30 seconds (Polytron) and centrifuged at 4,000 x g for 15 minutes. Supernatents were collected and dried under nitrogen gas. Extracts were reconstituted in 500 µl methanol. Subsamples of all serosal and mucosal extracts were dried and subjected to enzyme hydrolysis with 980 U β-glucuronidase and 9.1 U sulfatase (β-glucuronidase from Helix pomitia, Sigma Aldrich, G-0876) for 2 hours @ 37°C. Enzymatic reactions were stopped by the addition of equal volumes of methanol. The samples were centrifuged at 16,000 x g, dried under vacuum and reconstituted in 250 ml methanol prior to analysis. All samples were analyzed using reverse-phase HPLC as described above. The recovery of the flavone internal standard from the serosal solution and mucosal extracts was 90 ± 1%.

Statistical Analysis

All values reported are expressed as means ± standard error of the mean. Changes in body weights (final weight-initial weight) and ACF number, multiplicity and size were analyzed using one-way analysis of variance test (ANOVA) with a t-test as a post-hoc analysis for individual treatment comparisons. Daily food consumption was analyzed using a 2-way ANOVA for enzyme vs. alfalfa and treatment comparisons were made using a t-test.

Treatment comparisons for incidence of Rag detection in colonic extracts were made using a Fisher's Exact test. A chi-square test was used to detect significant difference in the incidence of Rag detection in the colons of mice between treatments. All other treatment comparisons were achieved by performing a 1-way ANOVA followed by a Tukey-Kramer post-hoc test. In the experiment conducted with the jejunal intestinal sacs, treatment comparisons were made across each sac section (mucosa and serosal solution). All statistical analyses were performed using SAS software (SAS Institute) and $P < 0.05$ was considered to be significant.

Results

PCR and HPLC analysis of transgenic alfalfa plants

PCR analysis of genomic DNA from fresh leaves confirmed the presence of the kanamycin gene in 43% (26/61) of attempted plant crosses made between transgenic Cimarron with non-transgenic Pioneer 5246. Plants with this gene were used as the TA for the studies reported here. The 99-4 transgenic parent plants produced a similar band whereas no fragment was amplified in the 5246 nontransformed parent plants (data not shown). The HPLC-UV chromatograms of leaf and stem extracts from plants which were positive for the kanamycin gene were found to contain an additional broad peak which coeluted with an authentic RG standard (Figure 2). The UV spectrum of the peak, which demonstrated a maximum absorbance at 308 nm, closely matched the spectrum of the RG standard (Figure 2, insert). The quantitative HPLC analysis of dry, ground plant material demonstrated that RG levels were 193 ± 17.58 µg RG/g dry weight (N=10) in the transgenic plants.

Diet Analysis

Diets were analyzed prior to and following five weeks storage at -20°C. RG, but not Rag, was identified in all TA diets supplemented with α-galactosidase or β-glucosidase. The amount of Rag in the BD+Rag diets following 5 week storage at -20°C was 84 ± 23 nmol Rag/kg diet (N=5), 84% of the amount added to the diets at the beginning of the study. The recovery of RG in TA diets following 5 weeks was 88% (N=10) (88 ± 21 nmol RG/kg diet).

The recovery of β-glucosidase activity in the diets supplemented with E1 and E2 following five weeks storage was 85% (731 ± 77 β-glucosidase U/kg diet) (N=10). The β-glucosidase activity of BD supplemented with E1 was also determined following incubation for 30 minutes at pH 3 and 7 to determine if the enzyme can remain stable under various conditions of the gastrointestinal tract. At pH 3, the activity of the BD with E1 was 267 ± 12 U/kg diet (N=5) (31% recovery of the original activity) and at pH 7, the activity of the diet was 430 ± 65 U/kg diet (N=5) (50% recovery).

Body weights and food consumption

The body weights of the mice did not differ between treatment groups at baseline (mean = 24.4 ± 1.3 g; p= 0.6). The mice gradually gained weight throughout the five-week duration of the study with the average gain being 4.2 ± 1.6 g, which was not significantly different between treatments (p=0.4). The addition of E1 or E2 to the diets did not significantly influence food consumption (p=0.11) (Figure 3). However, mice fed diets containing alfalfa consumed more food per day compared to mice fed BD with or without enzyme supplementation (4.3±0.1 g/day compared to 3.8±0.1 and 3.7±0.1, respectively. p<0.001 for both comparisons). Mice fed diets containing alfalfa with either E1 or E2 consumed, on average, 4.6 ± 0.1 g of food/day, which was significantly more than all of the other groups.

There was no difference in food intake between mice fed diets with CA verses TA (p=1.0, data not shown).

Formation of Aberrant Crypt Foci in the colorectal mucosa

The total number of ACF in the entire colon was significantly reduced in mice fed diets containing alfalfa relative to mice fed basal diet following the five-week, post-initiation experimental period (Table 1). This reduction was observed with both CA and TA. However, the number of ACF lesions/colon were reduced by an additional 64% in mice fed TA supplemented with E1 relative to mice fed TA without the enzyme (p=0.02). A similar reduction was observed with the addition of E2 to the TA, but the difference was not as statistically significant (p=1.2).

The addition of authentic Rag at 0.002% in the BD by dry weight resulted in a 74% reduction in the total number of ACF/colon in mice fed these diets compared to mice administered BD, alone ($p<0.0001$). Interestingly, the addition of authentic RG to BD in equal molar concentrations to RG in combination with E2 resulted in a similar reduction in the number of ACF/colon relative to mice fed basal diet (p=.0005). Diets containing RG, alone, without E2 supplementation, did not reduce ACF number. Furthermore, there was no difference between ACF numbers in mice fed basal diet with or without E2 or between mice fed CA with or without either E1 or E2. There was also no difference between multiplicity, overall (mean= 1.6±0.5 aberrant crypts/focus) and size of ACF, overall (mean = 179 ± 57 μm) between treatments.

Aberrant crypt lesions were primarily concentrated in the distal 4 cm of the colon (differentiated in 2 cm portions as the distal and middle region during our analysis). Few ACF were observed in the proximal 1 cm of the colon examined. Similar trends in the

number of total ACF between treatment comparisons were observed for the number of ACF in the distal and middle portions of the colon. The addition of E1 to TA reduced the number of ACF lesions in the distal colon by 55% ($p=0.06$) and by 72% ($p=0.09$) in the middle portion of the colon. The addition of E2 to diets containing authentic RG in BD resulted in a reduction of ACF number in the distal portion of the colon ($p=0.003$), but not significantly in the middle portion.

Colon and plasma concentrations of resveratrol following treatments

Rag could be detected at low levels (all under < 1 nmol/g tissue) in the colon of mice fed diets containing TA with or without E1 or BD with authentic Rag (Table 2). Liquid chromatography-MS/MS analysis confirmed the presence of Rag in the colon of CF-1 mice fed diets containing the TA (figure 4). Rag was identified by comparison with the retention time and the fragmentation pattern of the authentic standard.

As table 2 illustrates, tissue levels of Rag were similar in mice administered TA with E1 and mice fed Rag. Rag was also detected in the colon of mice administered TA without enzyme. However, the colonic Rag concentrations in the mice fed TA, alone, was about half that measured in the mice fed TA with E1 ($p=0.09$). Furthermore, the incidence of Rag detection was significantly less in the TA group relative to TA with E1 (60 vs. 100%, respectively, $p<0.05$). The colon concentrations of Rag in mice fed TA without E1 was also lower than concentrations measured in the Rag group ($p<0.05$). Resveratrol-aglycone was not detected in the colons of mice fed control, non-TA or CA and BD. The presence of Rag could not be confirmed in the plasma extracts regardless of β-glucuronidase/sulfatase enzyme digestion. RG could not be identified in any of the colon or plasma samples.

Resveratrol intestinal and colonic epithelial and plasma concentrations following gavage feeding

Rag could be detected in the colonic and intestinal epithelium cells of CF-1 mice fed a single 25 mg (110 µmol)/kg body weight oral dose of Rag via gavage at all time points examined (Figure 5A and 5B).The amount of Rag detected in the small intestine was significantly higher in extracts treated with β-glucuronidase/sulfatase (Rag + metabolites) relative to undigested extracts (figure 5A). In the small intestines, there was no difference in amount of Rag detected in β-glucuronidase digested extracts throughout the time-course. In contrast, there was no significant difference between the concentration of Rag detected in colonic epithelial extracts digested with β-glucuronidase and sulfatase vs. extracts analyzed without enzyme digestion (figure 5B). Colon epithelial concentrations of Rag were significantly higher 7 hours post-gavage relative to values obtained 1 hour following dose administration. Free, unconjugated Rag could not be detected in the plasma of mice administered Rag (figure 5C). The plasma concentration of glucuronide/sulfide metabolites of resveratrol were higher 7 hours post-gavage than at 3 hours in these mice.

In contrast to these findings, neither RG nor Rag was detected in the plasma or colon and small intestine epithelia of mice orally administered 25 mg (64 µmol)/kg RG at the time points examined.

Deglycosylation of piceid by lactase phlorizin hydrolase in small intestine

Everted-jejunum intestinal sacs from CF-1 mice were incubated with ^{14}C-lactose and 0-300 µM *N*-(*n*-butyl)-deoxygalactonojirimycin (NB-DGJ). The recovery of ^{14}C-glucose in the tissue and serosal solution of the intestinal sacs was significantly reduced with 10µM treatment of NB-DGJ (Figure 6) and was further reduced at a NB-DGJ dose of 300 µM. At

300 μM the recovery of ^{14}C-glucose was 47% of what was achieved in the control group. Consequently, this dose was used in the follow-up study to examine the role of lactase in the deglycosylation of RG in the jejunum.

To determine if LPH is involved with the deglycosylation of RG in the intestine, everted intestinal sacs from CF-1 mice were incubated in 100 μM RG in the presence or absence of 300 μM NB-DGJ. Following incubation in 100 μM RG for 15 minutes at 37°C, RG was detected in the mucosa and serosal solution of the jejunum (Table 3). Rag was only detected in the mucosa. In intestinal sacs incubated with 100 μM Rag, Rag was detected in the mucosa, but not the serosal solution. RG was not detected in any of the Rag treated samples. Subsamples of all mucosal and serosal extracts were digested with glucuronidase containing sulfatase to account for glucuronic acid or sulfate metabolites that may have formed. Rag was detected in all serosal solutions following enzyme treatment, indicating that complete phase-II metabolism of Rag had occurred (Figure 7). The concentration of resveratrol glucuronide/sulfate metabolites in the mucosa and serosal solution of intestinal sacs incubated with RG and NB-DGJ was significantly less than the concentrations detected in the mucosa and serosal solution of intestinal sacs in RG without NB-DGJ. The presence of NB-DGJ did not affect the concentrations of Rag or resveratrol-metabolites in the mucosa and serosal solution of intestinal sacs incubated in Rag.

The concentration of Rag in the mucosa of samples incubated with 100 μM Rag without NB-DGJ was almost five times greater than the concentration of RG without NB-DGJ in the mucosa of samples incubated with 100 μM RG. There was a similar difference between the concentrations of resveratrol-metabolites in the mucosa between intestinal sacs incubated with Rag without NB-DGJ and RG without NB-DGJ. The amount of resveratrol glucuronic

acid/sulfide metabolites in the serosal solutions of intestinal sacs treated with Rag was approximately two times greater than the concentration of metabolites in RG-treated sacs.

Discussion

Studies have shown that Rag may have protective activity against colorectal cancer (Tessitore et al, 2000; Sengottuvelan, 2006a; Sengottuvelan, 2006b; Sengottuvelan, 2006c). Previously, we reported that TA that accumulates resveratrol as RG inhibited the number of AOM-induced ACF in CF-1 mice when supplemented with α-galactosidase (E1), but not without enzyme supplementation (Kineman et al, submitted). To our knowledge, no other studies have investigated the bioactivity of RG against colorectal cancer. Our hypothesis was that RG was not bioavailable and the glucosidase activity of the E1 liberated Rag from RG in the TA resulting in the reduction of ACF in the colon.

In the present study we show that the addition of E1 to the TA resulted in a 64% reduction in the number of AOM-induced ACF in CF-1 mice fed these diets compared to mice fed diets with TA alone. The addition of β-glucosidase (E2) to TA resulted in a very similar reduction, suggesting that the glucosidase activity of E1 in combination with RG accumulation was responsible for the ACF reduction in these mice. Additionally, the supplementation of authentic RG to control diets had no influence on ACF number whereas the addition of E2 to these diets resulted in a 41% decrease in the number of ACF in mice who ate these diets compared to control-fed mice. This reduction was similar in magnitude to that achieved in mice administered control diets with Rag at equal molar concentrations to the RG contained in the these diets. These findings support the hypothesis that RG was not as bioavailable as Rag.

In contrast with our previous study (Kineman et al, submitted), alfalfa, independent of RG-accumulation, reduced the number of ACF in the colon of CF-1 mice in the study reported here. This was probably due to the different alfalfa cultivars used between the two studies. It is possible that the plants in the current study accumulate additional health-promoting compounds, or the same chemicals, but in higher concentrations, than the plants used in our previous study. Alfalfa normally accumulates a number of secondary metabolites that may have health-promoting activity (Stochmal et al, 2000a; Stochmal et al, 2000b). It is possible that the phytochemical profiles of the two cultivars differ, but this was not examined in our studies. In spite of this, the addition of E2 to the TA further enhanced the protective activity against AOM-induced ACF in CF-1 mice in comparison with transgenic alfalfa alone.

Since β-glucosidases occur ubiquitously in plants, being localized in the cell wall of dicot plants, such as alfalfa (Esen, 1993), we measured the endogenous β-glucosidase activity contributed by the alfalfa. We also explored a strategy to induce the release of endogenous β-glucosidases from the alfalfa through chopping fresh plants prior to processing the material through drying and milling. However, this did not have any affect on the endogenous β-glucosidase activity of the plant material after processing (mean= 0.117 ± 0.076 glucosidase U/g of dry material; p=0.8). Based on our measurements, alfalfa, when added at 20% in the diet, accounted for 23.4 β-glucosidase U/kg of diet, whereas exogenous β-glucosidase was supplemented in the diets at 860 β-glucosidase U/kg of diet. Thus, the alfalfa plants contribute some β-glucosidase activity, but at a very minor level compared to the amount we supplemented in the diets.

We tested the hypothesis that piceid from the TA is not bioavailable by quantifying by LC/MS the amount of Rag present in the colon and plasma of the mice following the 5-week treatment period. The amount of Rag found in the colon was below 0.5 nmol/g of tissue in all samples where it was identified. Rag was detected in the colon of mice fed TA without E1, which suggests that deglycosylation of RG does occur somewhere along the digestive tract. The concentration in these mice tended to be less than mice fed TA with E1 ($p=0.09$). Rag was also detected in fewer of these animals relative to the animals fed TA with E1 ($p<0.05$, Fisher's Exact Test). RG was not detected in any samples, indicating that it is not absorbed intact in the colon.

We hypothesize that the supplemented β-glucosidase activity was effective in liberating Rag from RG in the diets during the process of digestion and not prior to this, since Rag was not detected in the TA diet preparations with supplemented E2. We were also able to verify that the enzymes can retain some β-glucosidase activity at a pH of 3 and 7, which would indicate that the enzyme could be active under the pH conditions of the mouth, stomach and lower gastrointestinal tract.

Since the epithelium of the colon is the site where the majority of colorectal cancers originate, we compared the concentrations of Rag and RG that can reach this tissue following the administration of high doses of both compounds. At a dose of 25 mg *trans*-Rag/kg body weight, Rag was detected at low levels (< 2 nmol/mg protein) in the colonic epithelium 1, 3 and 7 hours post-gavage in the CF-1 mice. Higher levels were observed in the small intestine epithelium following β-glucuronidase/sulfatase treatment of these extracts, indicating that phase-II metabolism of the aglycone occurred at this site. Resveratrol was not detected in the aglycone form in the plasma. Contrary to these findings,

neither RG nor Rag could be detected in the plasma or epithelial of the colon or small intestine at the 1, 3 or 7 hour post-gavage time points of mice that were administered 25 mg RG/kg body weight. Lv et al (2006) recently reported that peak plasma levels of approximately 0.9 μM RG were reached within 20 minutes in Wistar rats orally administered 50 mg RG /kg body weight and rapidly declined within an hour. Unfortunately, comparisons with Rag were not made in that study. Our findings would suggest that the bioavailability of RG was much less than that of the Rag. Also, since neither RG nor Rag could be detected in samples at later time points in mice intragastrically fed a high dose of RG, this suggests that the systemic metabolism of RG may be different than that of the Rag.

Emerging evidence indicates that LPH may play an important role in the bioavailability of polyphenolic-glucosides (Day et al, 2000 and 2003; Nemeta et al, 2003). Since LPH is an extracellular enzyme located at the apical surface of the small intestine and has β-glucosidase activity, it could potentially catalyze the cleavage of glycosidic linkages in polyphenolic compounds resulting in the release of aglycone compounds that may be absorbed intact in the intestine or become available to the colon if unabsorbed in the upper gastrointestinal tract.

We further examined the role that LPH may play in the deglycosylation of RG in the intestines by conducting a transport study in everted, jejunal sacs from CF-1 mice using a lactase-specific inhibitor NB-DGJ. Our study suggested that LPH can deglycosylate RG to Rag, since the presence of NB-DGJ reduced the recovery of resveratrol-glucuronide/sulfate metabolites in the mucosa and serosal solution of the intestines. This data is in agreement with the observations made by Henry-Vitrac et al (2006) in Caco-2 cells, showing that deglycosylation was reduced in the presence of lactase-specific inhibitors. RG was also

detected in both the mucosal and serosal samples, indicating that it can be absorbed intact in the small intestines.

Our data revealed that Rag is either transported more efficiently across the intestinal wall or accumulates at higher concentrations in the mucosa compared to RG. The concentration of Rag detected in the mucosa of the intestinal sacs following incubation in 100 μM was almost 40-fold higher than the amount of Rag and almost 5-fold greater than the concentration of RG detected in the mucosa of sacs incubated in equal molar concentrations of RG. In a comparative study, Rag was shown to be transported at a higher rate and in greater amounts than RG into the human Caco-2 colorectal cells (Henry et al, 2005).

The resveratrol detected in the serosal solutions of intestinal sacs incubated in 100 μM Rag was exclusively in the form of glucuronic acid or sulfate metabolites. Glucuronic acid or sulfate metabolites were also a large fraction of the resveratrol detected in the mucosa samples in these intestinal sacs. These observations are in strong agreement with other studies showing that phase-II metabolism of Rag occurs in the small intestine (Andlauer et al, 2000; Kuhnle et al, 2000). The concentration of glucuronide/sulfate metabolites of resveratrol in the serosal solutions of intestinal sacs incubated in Rag without NB-DGJ was approximately 50% greater than the concentration of metabolites detected in the serosal solution of intestinal preparations incubated in RG, without NB-DGJ, further indicating that the absorption of Rag in the intestine may be more efficient than that of RG.

RG that passes through the upper gastrointestinal tract (g.i.) undigested could be hydrolyzed in the lower g.i. tract by microflora β-glucosidases. This has not been confirmed in any studies, to our knowledge. However, studies conducted in ileostomy patients indicate that the colon bioavailability of some polyphenol-glucosides may not be very high, since

these compounds are either largely absorbed or metabolized in the small intestine (Hollman et al, 1995; Kahle et al, 2006). Thus, the majority of ingested RG may be absorbed in the upper g.i. tract and does not reach the colon.

Bioavailability studies reveal that the gastrointestinal tract accumulates much higher amounts of Rag following oral administration of the compound relative to other tissues (Vitrac et al, 2002 and El-Mohsen et al, 2006). We were not able to detect Rag or RG in the plasma following the five-week treatments in the mice, which is in accordance with these studies and suggests that beyond the g.i. tract, the chemopreventive activity of resveratrol may not be achievable via physiologically relevant doses. However, few studies have attempted to link the bioavailability vs. bioactivity of resveratrol at a tissue following similar oral doses, so little is known about the potency of resveratrol in biological systems. Asesni et al (2002) reported that Rag following oral doses via gavage (20 mg/kg body weight daily) or the drinking water (25 mg/kg body weight) inhibited the hepatic metastatic invasion of B16 melanoma cells that were injected into the spleen of mice prior to the treatments. Following the 21 day study, Rag plasma levels were below 0.1 μM, which would suggest that in-vitro studies may not provide a real sense of the potency of Rag, since Rag concentrations below 5 μM rarely induce noticeable activity in cell culture systems (Gescher and Steward, 2003). In our studies, Rag, when administered at an approximate daily dose of 3 mg/kg body weight to CF-1, resulted in the significant reduction in the number of ACF in colons that averaged 0.2 nmol Rag per gram of wet tissue after a five-week treatment period. The dose of Rag in our study is still relatively high, considering that the most resveratrol-rich red wines contain about 15 mg Rag/L of wine. At this concentration, the mice in our

study received the equivalent of about 500 L of red wine/day, which raises some doubt on the feasibility of achieving clinically relevant doses of Rag via natural sources, alone.

In summary, our findings suggest that RG, the glucoside of Rag, may not be bioavailable to the colon. Although RG is a substrate for LPH in the intestine, the inability of TA or purifed RG to affect the number of AOM-induced ACF in CF-1 mice may be the result of the intact absorption of RG in the upper intestine and its subsequent rapid clearance from the systemic circulation or its limited metabolism in the colon. The addition of β-glucosidase activity to TA appeared to be effective in reducing ACF lesions by liberating Rag from RG. Since RG is the predominant form of resveratrol in natural sources and in other genetically-modified crops which over-express *resveratrol-synthase*, the human bioavailability of RG certainly warrants future investigation.

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Table 1: Azoxymethane-induced Aberrant Crypt Foci number, multiplicity and size in CF-1 mice

Treatment	Incidence	Size (μm) (p=0.2)	Multiplicity (AC/F) (p=0.4)	Total ACF (p<0.0001)	D	M	P
					#ACF (p<0.0001)	#ACF (p=0.002)	#ACF (p=0.28)
BD	9/20	198.9 ± 19.0	1.7 ± 0.1	6.3 ± 0.6^{A}	3.5 ± 0.4^{A}	2.5 ± 0.3^{A}	0.3 ± 0.1
BD+E2	8/9	209.8 ± 20.0	1.7 ± 0.2	5.7 ± 0.9^{A}	3.9 ± 0.6^{A}	1.4 ± 0.5^{B}	0.3 ± 0.2
BD+RG	8/10	166.3 ± 20.0	1.4 ± 0.1	5.5 ± 0.9^{A}	3.7 ± 0.6^{A}	1.4 ± 0.4^{B}	0.4 ± 0.2
BD+Rag	11/20	170.0 ± 17.1	1.5 ± 0.1	1.6 ± 0.6^{C}	0.9 ± 0.4^{B}	$0.7 \pm 0.3^{B,C}$	0.1 ± .01
BD+RG+E2	7/10	230.0 ± 21.4	2.0 ± 0.1	$2.6 \pm 0.8^{B,C}$	0.8 ± 0.5^{B}	1.5 ± 0.4^{B}	0.5 ± 0.1
CA	14/20	172.5 ± 20.0	1.6 ± 0.2	$2.3 \pm 0.6^{B,C}$	1.3 ± 0.4^{B}	$0.9 \pm 0.3^{B,C}$	0.1 ± 0.1
CA+E1	15/20	192.3 ± 18.9	1.8 ± 0.2	$2.3 \pm 0.6^{B,C}$	1.7 ± 0.4^{B}	$0.5 \pm 0.3^{B,C}$	0.1 ± 0.1
CA+E2	7/10	147.1 ± 21.4	1.4 ± 0.2	$2.8 \pm 0.9^{B,C}$	1.5 ± 0.6^{B}	$0.8 \pm 0.4^{B,C}$	0.4 ± .01
CA+Rag	11/15	181.4 ± 17.1	1.5 ± 0.2	$1.5 \pm 0.7^{B,C}$	0.9 ± 0.5^{B}	$0.6 \pm 0.4^{B,C}$	0.1 ± 0.1
TA	16/20	173.9 ± 20.0	1.7 ± 0.3	3.3 ± 0.6^{B}	2.0 ± 0.4^{B}	$1.1 \pm 0.3^{B,C}$	0.3 ± 0.1
TA+E1	11/20	158.6 ± 20.0	1.4 ± 0.2	1.2 ± 0.6^{C}	0.9 ± 0.4^{B}	0.3 ± 0.3^{C}	0.1 ± 0.1
TA+E2	6/10	148.3 ± 23.1	1.4 ± 0.2	$1.7 \pm 0.9^{B,C}$	1.1 ± 0.6^{B}	$0.4 \pm 0.5^{B,C}$	0.2 ± 0.5

Values represent means ± SE (N=9-20). Treatments abbreviations are defined as follows: BD=basal diet, CA= control alfalfa, TA=transgenic alfalfa, RG= piceid, Rag= resveratrol-aglycone, E1= α-galactosidase and E2= β-glucosidase. ACF number, multiplicity and size were assessed in three increments of the colon (distal most 2 cm (D), middle 2 cm (M) and proximal 1 cm (P)). Treatments assigned different letters within a column are significantly different ($p<0.05$; 1-way ANOVA, SAS).

Table 2: Concentration of resveratrol-aglycone (Rag) in colon of CF-1 following 5-week dietary treatments

Treatment	# where Rag was detected/total # assessed	Concentration (nmol/g tissue) in mice where Rag was detected
Basal diet (BD)	0/15	ND
BD + resveratrol-aglycone	13/15	0.27 ± 0.14^{A}
Control alfalfa (CA)	0/15	ND
CA + α-galactosidase (E1)	0/15	ND
Transgenic alfalfa (TA)	$9/15^{\dagger}$	0.10 ± 0.08^{B}
TA + E1	15/15	$0.22 \pm 0.18^{A, B}$

Values represent means ± SD (N=15). Female CF-1 mice were administered dietary treatments for 5 weeks. The transgenic alfalfa diets contained 100 ± 9 µmols piceid/kg diet. Resveratrol-aglycone was supplemented to basal diet in equal molar concentrations (100 µmols/kg diet) to the piceid in the transgenic alfalfa diets. Colon extracts were analyzed by liquid-chromatography-MS for the presence and amounts of resveratrol-aglycone and piceid. Values with different letters are significantly different ($p<0.05$; Tukey-Kramer). † symbol indicates that the incidence of Rag detection in the colon of mice fed TA diets was significantly less than that of mice fed TA + E1 diets ($p < 0.05$; Chi-square test). Piceid was not detected in the colon extracts. ND indicates that resveratrol-aglycone was not detected.

Table 3: Deglycosylation of piceid by lactase-phlorizin hydrolase in jejunum of CF-1 mice.

			Concentration (nmol/g tissue)	
Treatment	Tissue	Form Detected	-NB-DGJ	+NB-DGJ
RG (100 µM)	Mucosa	RG	17.94 ± 2.7^{X}	17.2 ± 2.7
		Rag	2.3 ± 0.6	0.5 ± 0.2
		Metabolites	15.3 ± 8.4^{A}	4.8 ± 2.0^{B}
	Serosal solution	RG	8.8 ± 2.0	13.3 ± 2.7
		Rag	ND	ND
		Metabolites	$20.7 \pm 3.0^{A, X}$	8.8 ± 2.8^{B}
Rag (100 µM)	Mucosa	RG	ND	ND
		Rag	88.1 ± 13.7^{Y}	94.0 ± 14.7
		Metabolites	38.7 ± 15.7	36.7 ± 11.1
	Serosal solution	RG	ND	ND
		Rag	ND	ND
		Metabolites	40.2 ± 6.9^{Y}	35.0 ± 6.6

Values represent means ± SE (N=8). Everted, jejunum sacs were incubated with 100 µM *trans*-piceid or *trans*-resveratrol-aglycone (Rag) in the presence or absence of the lactase inhibitor *N*-(*n*-butyl)-deoxygalactonojirimycin (- or + NB-DGJ). Amount of piceid, Rag or glucuronide/sulfate metabolites of resveratrol (determined by measuring difference between Rag concentrations following digestion of extracts with β-glucuronidase/sulfatase from concentration of Rag+piceid before enzyme digestion) was assessed in mucosa and serosal compartment of intestinal sacs following the 15 minute incubation at 37°C. Concentration comparisons made in a row (A vs. B superscripts) within each tissue are significantly different; comparisons made in the –NB-DGJ column (X vs. Y superscrits) with each tissue are significantly different ($p<0.05$; Tukey-Kramer, SAS). ND indicates "not detected".

Figure 1: Calibration curve for the LC-MS analysis of *trans*-resveratrol. Negative ion mass spectrometry was used with selected ion monitoring detection. The correlation coefficient was determined to be 0.9973 over the range of 3- 4000 pmol.

Figure 2. HPLC chromatogram of transgenic alfalfa and control (non-transformed) alfalfa extracts. The reverse phase-HPLC chromatogram of methanol extracts of dried, powered alfalfa @ 308 nm. Extracts of alfalfa plants which were positive for the kanamycin gene revealed a peak with retention time at 13.1 minutes, consistent with an authentic piceid standard. The UV absorption spectra of the peak (insert) had a maximum UV absorbance of about 308 nm and matched that of the piceid standard (Apin Chemicals, UK).

Figure 3. Average daily amount of food consumed. Values represent means ± SE (N=19-60). +Enz refers to dietary treatments supplemented with 860 U β-glucosidase / kg of diet (in the form of α-galactosidase or β-glucosidase). + alfalfa indicates diets that contained either nontransgenic or transgenic alfalfa at 20% by dry weight. Dietary treatments assigned different letters signify groups that are significantly different ($p<0.05$; 2-way ANOVA, t-test, SAS).

Figure 4. LC/MS/MS spectrum of A). resveratrol-aglycone (Rag) standard and B). colon extract from mouse fed transgenic alfalfa with α-galactosidase (E1). Rag is identified by the retention time of the standard (16.1 minutes) and the fragmented ions m/z 208, 182 and 112.9.

Figure 5. Concentration of resveratrol-aglycone (Rag) in colonic and intestinal epithelium cells and

plasma of CF-1 mice following intragastric feeding of Rag (25 mg/kg body weight). Values represent means ± SE (N=5). Treatments with different letters differ significantly

($p<0.05$; Tukey-Kramer, SAS). Light bars indicate Rag (Rag detected without β-glucuronidase/sulfatase digestion). Resveratrol-aglycone was not observed in the plasma (panel C). Dark bars indicate Rag + resveratrol metabolites (resveratrol-aglycone detected following β-glucuronidase/sulfatase digestion). There was no significant difference between the amount of Rag detected in the colon extracts before and following β-glucuronidase/sulfatase digestion (panel B). On the other hand, free, unconjugated Rag could not be detected in the plasma samples.

Figure 6. Inhibition of ^{14}C-lactose deglycosylation by *N*-(*n*-butyl)-deoxygalactonojirimycin (NB-DGJ) in jejunum of CF-1 mice. Excised everted, jejunum sacs were incubated in the presence of 4.63 kBq/ml ^{14}C-lactose with various concentrations of the lactase inhibitor *N*-(*n*-butyl)-deoxygalactonojirimycin (NB-DGJ) for 15 minutes @ 37°C. Values represent means ± SE (N= 5).

Figure 7. HPLC Chromatograms of serosal extracts from intestinal sacs incubated with 100 uM resveratrol-aglycone. A). Untreated extract. B) Extract digested with β-glucronidase/sulfatase.

Figure 1

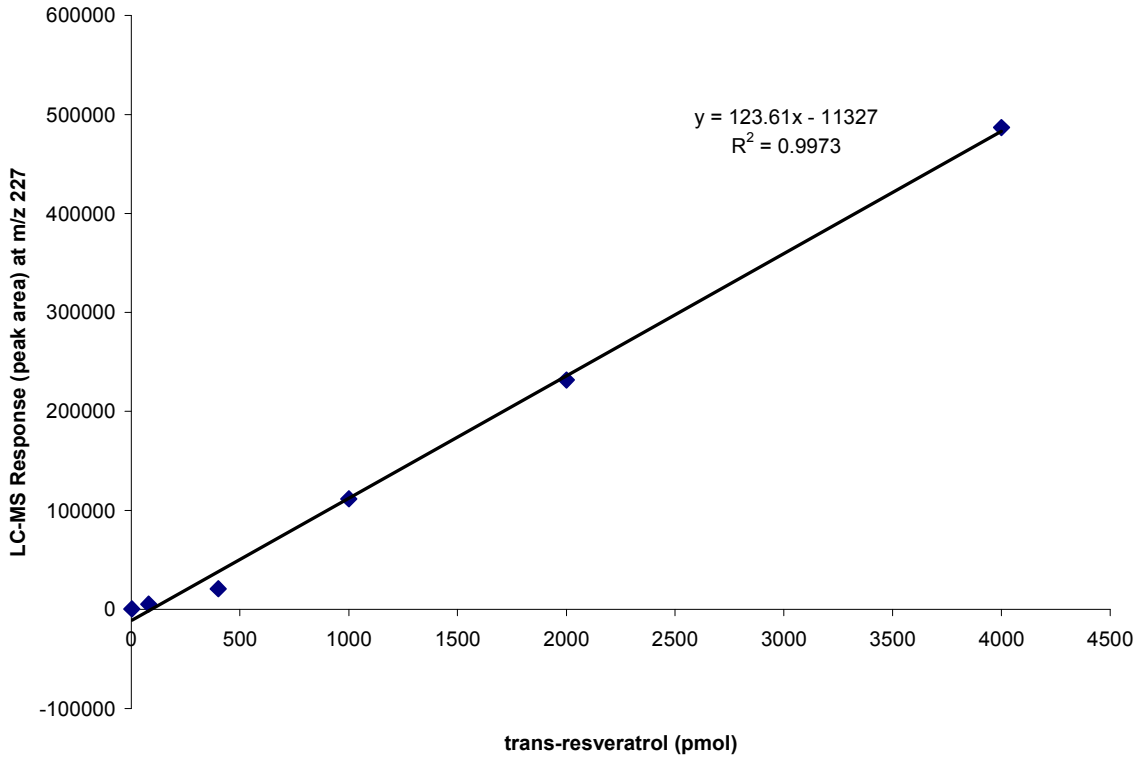
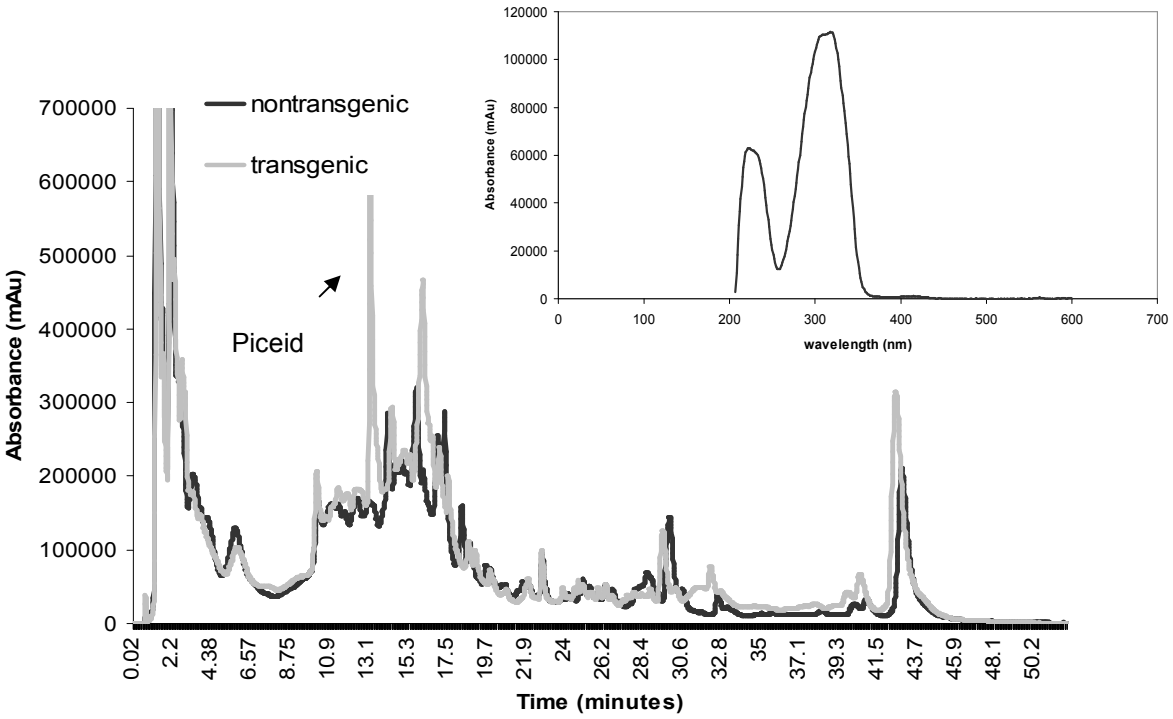


Figure 2.



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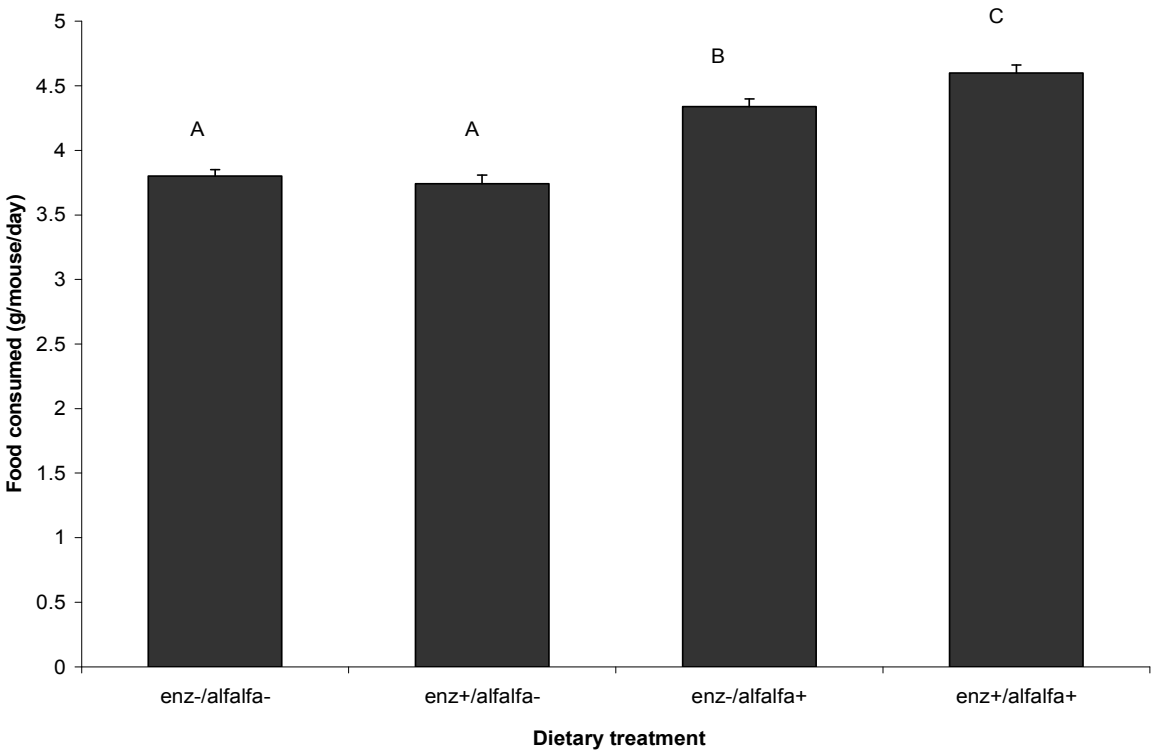
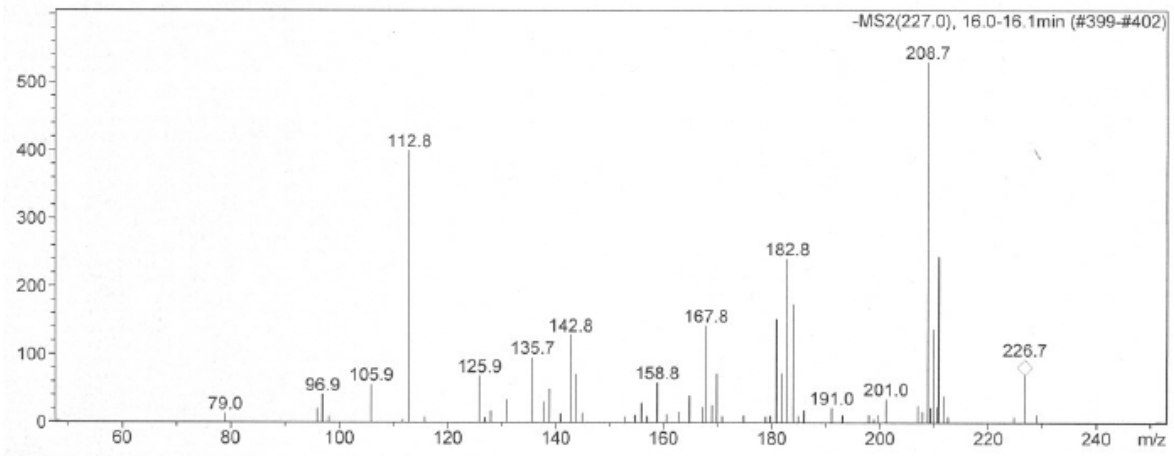


Figure 4.

A)



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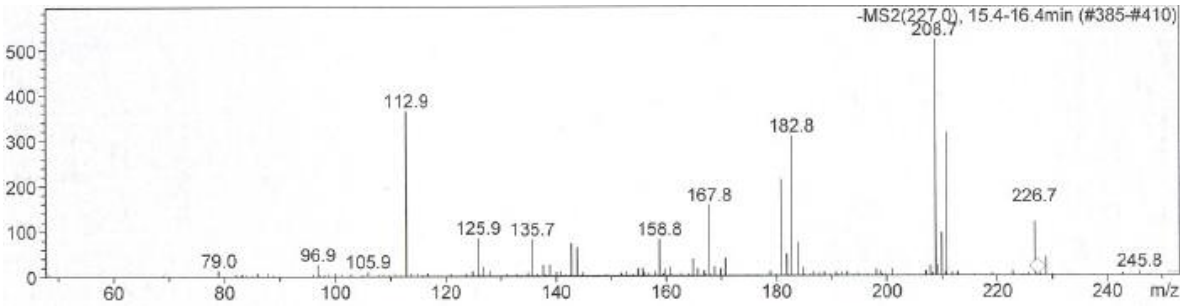


Figure 5.

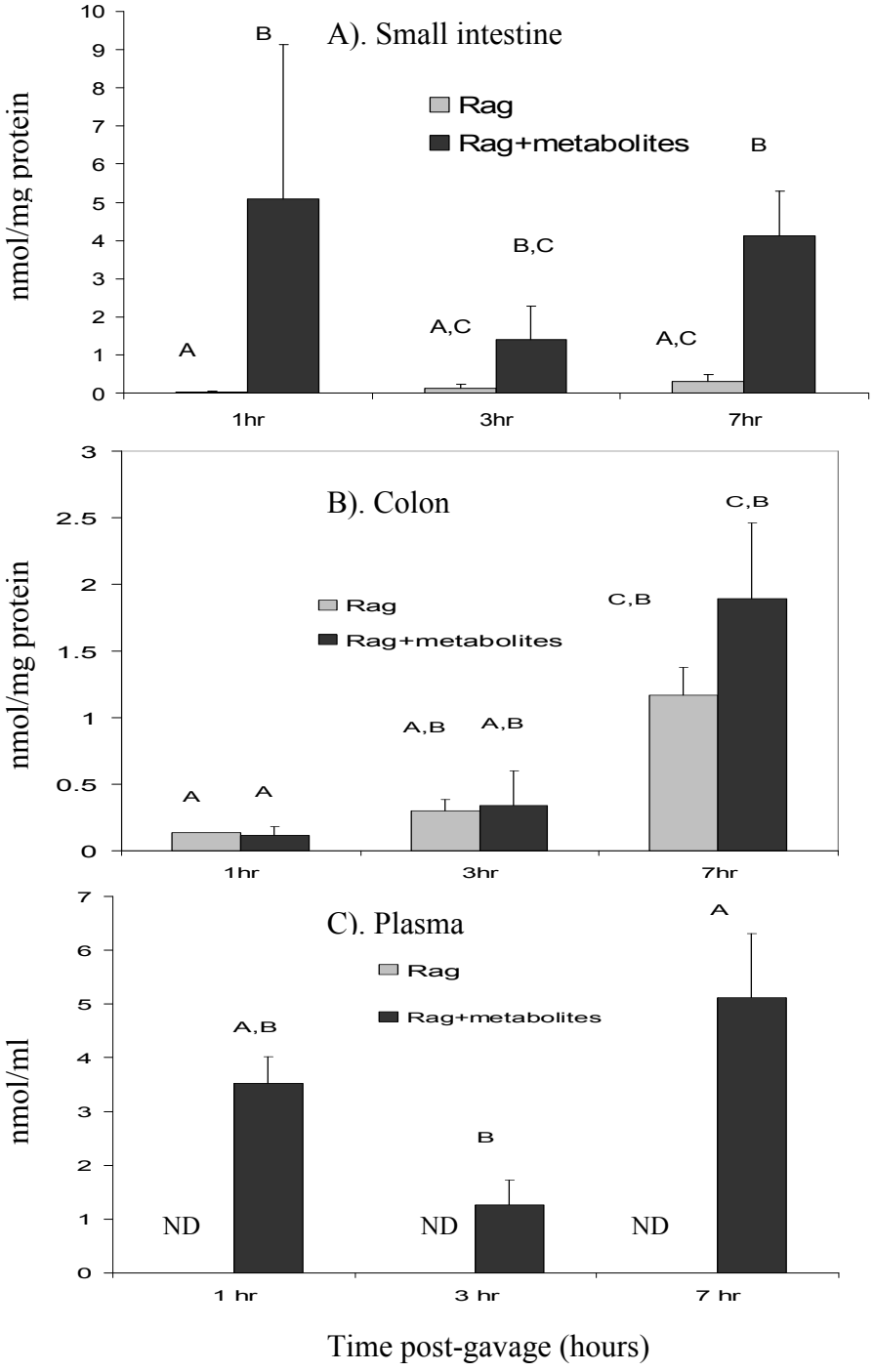


Figure 6.

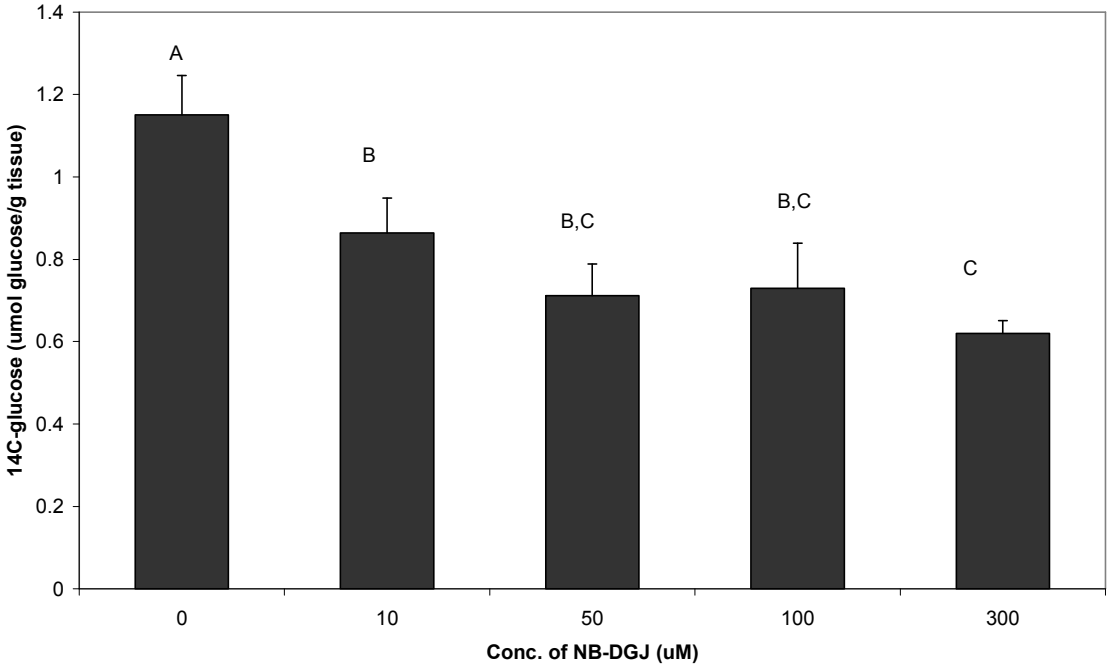
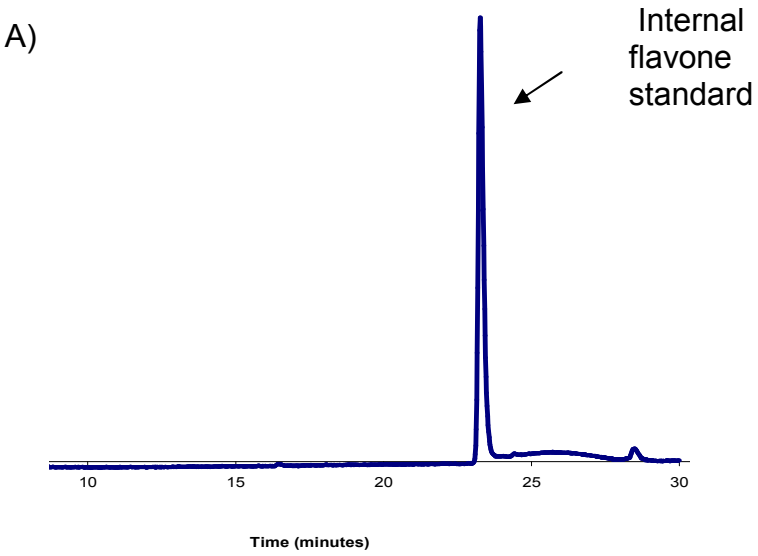
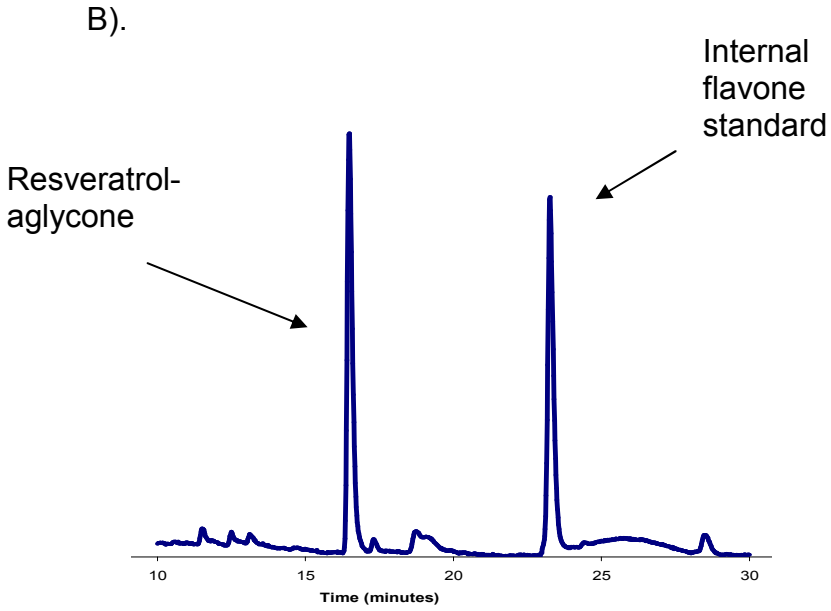


Figure 7.





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CHAPTER 4: GENERAL CONCLUSIONS

General Discussion

Resveratrol may be one of the most versatile botanical phytochemicals known, possibly possessing a myriad of biological activities. Recent studies suggest that resveratrol may alleviate diabetic complications (Szkidelski, 2006), protect against Alzheimer's disease (Riveire et al, 2006) and mimic the biochemical changes associated with calorie restriction (Baur et al, 2006). Unfortunately, the typical human diet is extremely limiting in resveratrol-containing foods (Cassidy et al, 2000; Burns et al, 2002), making it doubtful that clinically-relevant doses of resveratrol can be achieved through natural sources, alone. Metabolic engineering strategies in plants have been very successful in introducing disease resistance traits into new crops through the introduction of stilbene synthase genes and consequent biosynthesis of resveratrol in these crops (discussed in Literature review). This year alone, new reports emerged showing that resveratrol was introduced into apple (Ruhmann et al, 2006), lettuce (Liu et al, 2006) and peas (Richter et al, 2006). These crops, if introduced onto the commercial market, could provide an increased, more diverse pool of resveratrol-rich products as well as offer producers with more agronomical crop varieties. As with nontransgenic, natural sources of resveratrol, these crops accumulate resveratrol as piceid.

Our findings indicate that the bioavailability of piceid may be limited compared to resveratrol-aglycone, which raises questions on the feasibility of using GM products or natural products of resveratrol for chemopreventive or therapeutic purposes. We demonstrated that piceid-accumulating alfalfa, alone, did not affect the number, multiplicity or size of azoxymethane-induced aberrant crypt foci in the colon of CF-1 mice whereas the supplementation of transgenic alfalfa with exogenous β-glucosidase activity, via α-

galactosidase or purified β-glucosidase, did significantly reduce the number of AOM-induced ACF in these mice. Furthermore, we demonstrated this trend with an authentic piceid in basal diet, indicating that the protective effect observed with the β-glucosidase supplementation was not simply the result of piceid being liberated from the plant matrix. Liquid chromatography-mass spectrometry data of colonic extracts verified that the amount of resveratrol-aglycone that reached the colon of mice fed transgenic alfalfa with supplemented β-glucosidase activity for 5 weeks was greater than the concentration in the colon of mice fed transgenic alfalfa, alone ($p=0.09$). Piceid could not be detected in the colon of any of these mice, which further supports our hypothesis that piceid was not bioavailable to the colon. Since resveratrol-aglycone could not be detected in the plasma following the 5-week treatments, our results further imply that the systemic distribution of resveratrol-aglycone may not be very high.

The metabolic fate of polyphenolic-glucosides following oral administration is a controversial topic and, until recently, subject to much speculation since few bioavailability studies have been conducted on these compounds. The traditional hypothesis was that these compounds were very unlikely to be absorbed intact in the gastrointestinal tract, due to their larger, bulkier structure and polarity (Walle, 2004). Comparative studies between polyphenolic-aglycones and their glucosides reveal that the small intestine may play a much more significant role in the bioavailability of polyphenolic-glucosides than previously realized (Nemeta et al, 2003). Studies in ileostomy patients showed that quercetin glucosides were almost completely hydrolyzed and absorbed as aglycones in the small intestine of humans (Walle et al, 2000). Day et al in a series of classical studies, demonstrated that the intestinal enzyme lactase-phlorizin hydrolase (LPH) was capable of hydrolyzing quercetin-

glucosides (Day et al, 2000; Day et al, 2003), with quercetin-4-glucoside being a better substrate for LPH than quercetin-3-glucoside, indicating that the position of the glucose moiety on the parent compound may dictate the substrate suitability of the compound for LPH and, perhaps, the overall bioavailablity of the compound. LPH has since garnered special attention for its potential role in intestinal polyphenol metabolism, since it is located extracellularly along the brush boarder membrane of the intestine (Nemeta et al, 2003). Other intestinal β-glucosidases seem to be located in the cytosol of epithelial cells. Therefore, in order for polyphenol glucosides to be substrates for cytosolic β-glucosidases, they would initially have to be transported from the intestinal lumen across the lipid bi-layer membrane of the epithelial cells. Vitamin B-6 (Amanda et al, 2002; Mackay et al, 2004) as well as a variety of flavonoid and isoflavone compounds (Day et al, 2000; Day et al, 2003; Nemeta et al, 2003) have been shown to be possible substrates for LPH.

Our data demonstrate that piceid can be a substrate for LPH and be absorbed intact in the small intestines. These observations are in agreement with studies conducted in Caco-2 cells showing that piceid could be absorbed intact in addition to being deglycosylated by LPH (Henry et al, 2005; Henry-Vitrac et al, 2006). The sodium-glucoside cotransporter-1 (SGLT1) may play a role in the active transport of piceid into the intestinal epithelial, as has been demonstrated in Caco-2 cells using inhibitors of SGLT1 (Henry et al, 2005). However, these studies also indicate that piceid that is absorbed in the cells can be deglycosylated by cytosolic β-glucosidases (Henry-Vitrac et al, 2006). In contrast to this last finding and in agreement with our results, a recent study on the systemic distribution of piceid in Wistar rats showed that piceid was detected in the plasma and in various organs, such as the spleen,

lung, liver and brain in animals orally administered a high dose of piceid (50 mg/kg body weight) (Lv et al, 2006).

Our results suggest that the deglycosylation of piceid in transgenic alfalfa occurs somewhere along the digestive tract as revealed by the detection of resveratrol-aglycone in the colon of mice fed these diets. The human lower gastrointestinal tract contains about 10^{14} bacteria of more than 500 distinct species (Klein et al, 2006). The colon microflora express β-glucosidase activity that can hydrolyze glucoside compounds which pass through the upper g.i. tract, unabsorbed or undigested. However, colonic metabolism of some polyphenolic compounds does not simply end with hydrolysis of the sugar bonds. The microflora of the colon can further degrade polyphenolic compounds into a variety of phenolic and carboxylic acid products (Gontheir et al, 2003; Rechner et al, 2004) as largely reported for flavonoids. The structure of flavonoids and position of glycosidic bonds may dictate whether a flavonoid-glycoside will be a further degraded by colonic bacteria (Simons et al, 2005). Our study in the everted, jejunal sacs suggested that the absorption of resveratrol-aglycone is higher than piceid in the small intestine; therefore, it would be expected that most of the piceid would pass through into the colon. However, the metabolism of piceid in the colon seems to be unknown. In rats administered tritium-labeled resveratrol, there was no evidence that resveratrol-aglycone was further degraded to other compounds in these animals (El-Mohsen et al, 2006). Also, in a study were the human fecal water content of polyphenols was assessed, resveratrol-aglycone could be detected at a mean concentration of 0.04 μM, indicating that resveratrol can reach the colon, intact, in humans (Jenner et al, 2005). However, this study consisted of only 5 healthy, adult subjects who were on a non-restrictive diet, so the amount of resveratrol or piceid consumed by the

individuals was not known. It would be expected that the piceid which reaches the colon would be deglysoylated by colon bacteria and then have the same metabolic fate as that of resveratrol-aglycone from the colon. Our studies suggest that resveratrol-aglycone can reach the colon, but it is not known if degradation of at least some of the compound occurs at this site. At this time, the biochemistry of resveratrol-aglycone and piceid in the colon remains a black box.

It is possible that the majority of the piceid consumed in the diet via the transgenic alfalfa was metabolized in the small intestine. In a recent study between 0-33% of polyphenols in apple juice administered orally to ileostomy patients reached the end of the small intestines after 2 hours (Kahle et al, 2006). The type of sugar moiety attached to the polyphenol seemed to dictate the colon bioavailability of that molecule as a smaller percentage of glucosides were recovered at the distal end of the small intestine relative to other conjugated compounds and aglycones. This would suggest that the majority of piceid could possibly be metabolized or absorbed in the upper gastrointestinal tract, with a small percentage of the oral dose ultimately reaching the colon. In addition to the small intestine and colon, recent data has indicated that deglycosylation of glucosides can occur in the mouth (Walle et al, 2005). Regardless of the possible mechanism involved, our studies suggest that piceid may be bioavailable to the colon as resveratrol-aglycone, but the amount of the aglycone that ultimately reaches the colon from an oral dose of piceid was less than the amount that accumulated at the colon from an equivalent oral dose of resveratrol-aglycone.

In our studies, exogenous β-glucosidase enzymes were supplemented into diets with transgenic alfalfa as a strategy to liberate resveratrol-aglycone from piceid in these diets.

Although the exogenous β-glucosidase supplementation to piceid-accumulating transgenic alfalfa reduced the number of AOM-induced ACF in CF-1 mice, these studies would need to be followed up in a longer-term tumor experiment in order to provide a more complete assessment of the chemopreventive potential of this strategy. In a recent study, the number of AOM-induced ACF in F344 rats administered 10 g/kg body weight quercetin did not correlate with tumor development as quercetin decreased tumor incidence but had no affect on ACF development (Dihal et al, 2006). Rutin (quercetin-3-O-β-rutinoside) had no affect on tumor or ACF incidence. Plasma concentrations of 3-methyl-quercetin (the main metabolite of quercetin, identified) reached only 30% in rats administered 40 g rutin/kg body weight of what was observed in rats dosed with 10 g quercetin/kg body weight after 38 weeks, illustrating that the difference in anti-tumor activities between the two compounds may be related to the low bioavailability of rutin.

In our case, there were challenges in producing enough alfalfa to carry out a thirty-week tumor study, since the availability of alfalfa was limited due to its poor growth performance in the field and greenhouse. The ACF identified in our last study were counterstained with alcian blue to differentiate lesions that were depleted in the carbohydrate mucin. Mucin-depleted foci have been purported to be a subcategory of ACF that are more likely to be dysplastic and, thus, more likely to correlate with tumor development (Mori et al, 2005). MDF could only be identified in a few mice in that study and could not be fully assessed relative to dietary treatment due to the low statistical power.

Another limitation to our studies may be the use of the mouse model in examining the bioavailability of glucoside compounds. To our knowledge, it is not known how the intestinal activity of LPH in CF-1 mice compares with that of normal, healthy humans. It is

documented that the activity of LPH in Caco-2 cells is significantly less than lactose-intolerant adults (Chantret et al, 1994), so interpretations on the bioavailability of piceid using this model may not be accurate. If the activity of LPH is significantly higher in humans than mice, then piceid may be largely deglycosylated in the small intestine by LPH and absorbed as the aglycone in humans and would therefore be expected to be metabolized and distributed the same as resveratrol-aglycone. Also, the colonic microflora population may substantially differ between CF-1 mice and normal humans.

The fecal and cecal contents of the CF-1 mice fed the diet treatments for five weeks could have been assessed for piceid and resveratrol-aglycone concentrations to more fully assess the intestinal absorption and colonic metabolism of the two compounds. If piceid was detected in the colonic and cecal contents of mice fed purified piceid or transgenic alfalfa alone, then that would have suggested that the low colon bioavailability of piceid was largely the result of its poor absorption at that tissue and its limited deglycoyslation by the gut microflora. On the other hand limited detection of piceid in these samples would indicate that piceid was degraded in the colon or was largely absorbed intact in the small intestine and was metabolized differently than resveratrol-aglycone. For instance, resveratrol-aglycone can be redistributed to the intestine via the bile duct following phase-II conjugation in the liver (Marier et al, 2002). It is not known if piceid is hydrolyzed by liver β-glucosidases and further metabolized in a similar manner to resveratrol-aglycone.

The collective findings of our studies could spawn future investigations to address important questions on the bioavailablilty of piceid. For starters, the role of the sodium-glucose co-transporter-1 (SGLT1) in the intestinal absorption of piceid could be examined using a similar approach to the one we employed using everted, jejunal sacs from mice to

study the involvement of lactase in the deglycosylation of piceid. It would also be interesting to see if the presence of other plant constituents (i.e. polyphenolic compounds) influences the intestinal absorption of piceid, due to substrate competition for LPH or SGLT1. These comparisons could be made using the everted, jejunal sacs and Caco-2 cells with alfalfa extracts and pure compounds.

The rat-linked model designed by Marier et al (2002), where the bile duct of one rat is surgically linked to the duodenum of a recipient rat, could be used to study the potential of enterohepatic recirculation of piceid. The enterohepatic reabsorption of piceid in this model would be confirmed by the appearance of piceid in the plasma of the bile recipient rat following the oral administration of piceid to the bile donor rat.

In order to fully assess the potential of piceid as a nutraceutical, bioavailability of piceid could be more fully explored in a human study. It would be interesting to compare the plasma kinetics of piceid that is administered both orally and intravenously to resveratrol-aglycone introduced by the same routes at equivalent molar doses. Liquid chromatography/mass spectrometry analysis of plasma, urine and fecal samples could be used to identify resveratrol-metabolites and possible byproducts formed from the catabolism of resveratrol. The gut metabolism of piceid and resveratrol-aglycone could be more fully examined in humans by incubating both compounds in fecal samples to determine if piceid is a substrate for microflora β-glucosidases and degradation of the free, aglycone occurs in the colon.

One of the more interesting observations made in our studies was the different affect that the two alfalfa cultivars had on the formation of ACF in the colon of CF-1 mice. The studies illustrated in chapter 2 of this dissertation were conducted using the Regen SY alfalfa

germplasm. These studies did not reveal that the alfalfa, alone, offered any protective activity against ACF formation. On the other hand, the germplasm utilized in the later studies, Pioneer 5246, which were reported on in chapter 3, did reduce ACF number. Regen SY was originally selected as a laboratory strain (Bingham, 1991). This cultivar proved to be a poor performer under both outdoor and greenhouse conditions. The plants used in chapter 3 studies were grown under greenhouse conditions, exclusively, with superior growth performance to Regen SY. Although, there were no noticeable phenotypic changes in the transformed Pioneer 5246 plants relative to the transformed Regen SY, with the exception of growth, it would be interesting to see if the plants used in the later studies expressed higher levels of flavonoid or flavonoid-related compounds relative to the Regen SY plants used in the earlier studies. Cell-culture experiments using extracts generated from the plants could be conducted with comparisons to purified piceid and resveratrol-aglycone to examine the anti-cancer properties of the plants and any added activity that may be attributed to other compounds present.

In addition to alfalfa, the variety of other crops developed to express *stilbene- synthase* could possibly demonstrate health-benefiting properties. Some of these other crops could offer a variety of foods that could be processed to hydrolyze piceid to resveratrol-aglycone in the final products. Efforts could also be directed into improving the piceid content of alfalfa sprouts, through the use of alternative promoters. However, this may be seriously challenged by the high expression of flavonoid compounds in these tissues and, thus, possible substrate competition between *stilbene synthase* and *chalcone synthase*.

Since low colon concentrations of resveratrol (<0.5 nmol/g wet tissue) were associated with a reduction of AOM-induced ACF in the mice of our study, resveratrol may be a very

biologically potent compound. The extent of resveratrol's activities may not be fully illustrated in cell-culture studies, since resveratrol-aglycone doses below 5-10 μM rarely result in observable biochemical or molecular changes in *in-vitro* studies (Gescher and Steward, 2003). Thus, there is a need for studies linking tissue concentrations achieved with lower doses in *in-vivo* studies to doses required to achieve equivalent intracellular concentrations in *in-vivo* studies. Furthermore, additional studies examining the biological activity and bioavailabilty of resveratrol at specific tissues need to be examined.

In conclusion, our studies suggest that the colon bioavailability of resveratrol-aglycone from piceid is very low and indicates that chemopreventive activity at the colon by piceid is very limited. Our studies support a role for LPH in the intestinal metabolism of piceid and, thus, add to the accumulating literature suggesting that enzymes and transporters important in carbohydrate metabolism may also play a very important role in the metabolism of plant polyphenolic compounds. Furthermore, we provide evidence that GM strategies to increase concentrations of resveratrol in plants may provide health benefits to humans; however this strategy may be challenged by the limited bioavailability of piceid.

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