Cancer chemoprevention with flavonoids from cereal grains and legume

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Cancer Chemoprevention with Flavonoids from Cereal Grains and Legumes

By

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Date
Epidemiological studies have shown that approximately 50% of cancer incidence, and 30-35% of the cancer related mortality in Americans is associated with lifestyle habits, such as diet, tobacco and alcohol use. There is a direct relationship between diets rich in fruit and vegetables and a lower incidence of cancer. Polyphenols and flavonoids from green tea and soy have been extensively studied for their chemopreventive properties. However, cereal grains and legumes have not been well characterized even though they are a main component of the diet.

The objective of this study is to determine the possible chemopreventive properties of flavonoids and other polyphenols in cereal grains and legumes. Our approach towards chemoprevention is the inhibition of the carcinogenic process at the initiation step, by inducing Phase II enzymes, such as NADP(H): quinone oxidoreductase (NQO1), and at the promotion and progression step, by inhibiting cyclooxygenase-2 (COX-2).

In the present study, Hepalclc1c7 (murine hepatoma) cells were exposed for 48h to flavonoid extracts from cereal grains and legumes to evaluate their effect on NQO1 activity. Preliminary studies showed significant induction rates with most of the extracts. Experiments conducted with a different set of extracts showed a significant NQO1 induction with spring red wheat (1.5), winter wheat (1.6), wild rice (1.5), and lentil (1.8) extracts. Western blots were performed to assay COX-2 expression. Oats and rye flavonoids inhibited COX-2 expression in preliminary studies, after a 48 h exposure to U87 (human glioblastoma) cells. Studies with a second set of extracts failed to confirm these results. Further research is needed to evaluate dosage and toxicity. Collection of different fractions of the extractions should also be considered.
Abbreviations

AA: arachidonic acid
aFGF: acidic fibroblast growth factor
Ah: aromatic hydrocarbon
AP1: activator protein-1
ARE: antioxidant-responsive element
bFGF: basic fibroblast growth factor
BHA: 2(3)-tert-butyl-4-hydroxyanisole
BHT: 3, 5-di-tert-butyl-4-hydroxytoluene
COX: cyclooxygenase
DCPIP: 2, 6-dichlorophenolindophenol
ER: endoplasmic reticulum
ERK: extra-cellular signal-regulated kinase
FAP: familial adenomatous polyposis
GST: glutathione-S-transferase
HRP: horseradish peroxidase
I3C: indole-3-carbinol
LPS: lipopolysaccharide
MAPK: mitogen-activated protein kinase
NQO1: NAD(P)H: quinone reductase
NSAIDs: non-steroidal anti-inflammatory drugs
PG: prostaglandin
PGHS: prostaglandin endoperoxide H synthase
ROS: reactive oxygen species
TX: thromboxane
UDP-GT: UDP-glucuronyltransferases
VEFG: vascular endothelial growth factor
XRE: xenobiotic-responsive element
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INTRODUCTION

Cancer chemoprevention:

In Western societies cancer is one of the main causes of premature mortality, along with stroke and cardiovascular disease. The American Cancer Society estimates that 552,000 Americans died of cancer in the year 2000, and 1,220,100 new cancer cases were diagnosed (1). Epidemiological studies showed that nutrition and lifestyle factors, such as tobacco and excessive alcohol use account for approximately 50% of the cancer incidence and for 30-35% of the cancer related mortality (2). Although inherited genes influence the risk of developing cancer, genetic predisposition only explains a small portion of the total cancer cases. Cancer is a partially preventable disease and dietary and lifestyle choices can help to decrease the risk of cancer (1).

Cancer chemoprevention is the use of natural or synthetic compounds to block the development and progression of invasive neoplasia (3). Carcinogenesis is a multistep process that involves many biological alterations that can result in structural and functional changes in the genome. Carcinogenesis can be divided into three stages: initiation, promotion and progression. At initiation, a carcinogen interacts with DNA and causes simple mutations in the genome. DNA repair or metabolism of the initiating agents can alter the process of initiation, but if not repaired, it is considered an irreversible process because the genotype and/or the phenotype of the cells are established at this point. Initiated cells proliferate during promotion. Promotion is a reversible phase that occurs over a long period of time. Promoting agents alter gene expression through perturbation of signal transduction pathways, but they do not cause a direct alteration in DNA. Promoting agents can stimulate cell replication and inhibit
apoptosis. Although the exact mechanisms are unknown, the mitogen-activated protein kinases (MAPKs) pathway and the phosphorylation of cyclins may be involved. Progression is the phase where pre-malignant neoplasia is transformed into invasive cancer. The progression step of carcinogenesis is characterized by karyotypic instability, irreversible genetic alterations, and cell selection for optimal neoplastic growth (4) (reviewed in (5)).

Cancer preventive agents can be categorized in two main classes as blocking agents and as suppressing agents. Blocking agents act at the initiation step of carcinogenesis by preventing carcinogens to reach cellular targets. Some blocking agents prevent the metabolic activation of certain carcinogens. Others stimulate detoxification enzymes to metabolize carcinogens before they can reach their target sites. Suppressing agents act at the promotion and progression step of carcinogenesis. They prevent the evolution from pre-neoplastic cells to malignant cells (6, 7). Cancer preventive measures can be directed to high-risk groups or to the general population. For long-term assessment of populations at normal risk, the chemopreventive agents must be minimally toxic or nontoxic compounds. Dietary constituents are relatively safe and they are not perceived as medicine, which makes them the ideal candidates for this purpose. For the treatment of high-risk patients, those with genetic predisposition for certain cancers, previous cancer diagnosis or high exposure to a known carcinogen, compounds with a relative toxicity may be accepted (5, 8).

Nutrient imbalances are one of the main ways in which diet contributes to cancer development. These imbalances are reflected in western diets, which have a deficiency of fiber and excess of fat intake (2). Numerous epidemiological studies have shown that
dietary fiber and whole grains are related to lower incidences of certain cancers, such as gastric and colon cancer (9-11). In addition to the epidemiological data, animal studies showed a beneficial effect of fiber, especially wheat bran fiber, in colon and breast cancer (12, 13). The ideal fiber intake is 25-35 g/day in adults. Fifty percent of the total fiber intake should come from grains, 30% from vegetables and legumes, and about 20% from fruits (reviewed in (2)). One of the major nutritional excesses is the elevated proportion of calories of fat origin. In the United States fat intake accounts for 35% of the total calories, of which approximately 25% are saturated fatty acids. There is an association between high fat diets and elevated risk of developing colon cancer, breast cancer, prostate cancer, and lung cancer (reviewed in (2)).

As mentioned earlier, whole grains may have protective properties against certain types of cancer. Whole grains can be classified as major cereal grains including rice, wheat and corn, or as minor grains including barley, rye, oats, triticale, millet and sorghum (14). Wheat production constitutes one third of the total grain production and rice accounts for one fourth of the world production. Most grains are rich in fiber, starch, vitamins and minerals. They are low in fat and contain about 10-15 % protein. During milling, the bran and the germ are removed from the endosperm, which is then transformed into flour. In most refining processes, nutrients and phytochemicals present in high concentrations in the outer part of the grain are lost, reducing the nutritional value of the grain products (reviewed in (10)). Although most of their beneficial effects are related to their high content in fiber, other components such as antioxidants, phytoestrogens, phenolic compounds and trace elements may also play an important role in cancer prevention (10, 15). Whole grains contain significant amounts of phenolic
acids, mainly in the bran layer. In wheat bran, the principal phenolic acid is ferrulic acid (10). Caffeic and ferrulic acid prevent carcinogen activation and their reaction with cellular targets (7). Phenols extracted from the bran layer of brown rice inhibited cell proliferation and decreased clonogenicity in breast and colon cells (16). The potential cancer protective effect of phenolic compounds may be related to their ability to stimulate detoxification enzymes, to inhibit arachidonic acid metabolism and possibly, to their ability for chelating iron (10, 15). Other antioxidants present in whole grains are phytic acid, vitamin E and selenium. Phytic acid may be involved in the reduction of free radical production in the intestine by diminishing the amount of iron available for the Fenton reaction (15). Vitamin E is a fat-soluble vitamin that acts as a free radical scavenger. It has the potential to prevent the activation of carcinogens and to protect against oxidative damage in cellular membranes (6, 10). Selenium acts as a cofactor for glutathione peroxidase, an enzyme that catabolizes peroxides and prevents the formation of free radicals, protecting tissues from oxidative damage (reviewed in (3, 10)).

Along with cereal grains, legumes have been hypothesized as possible cancer chemopreventive agents. Legumes are the fruits or pods of a leguminous plant, such as beans, lentils and peas. Epidemiological studies have been inconclusive to show a protective effect against cancer with legumes (reviewed in (9)).

A variety of epidemiological studies have also shown a relationship between individuals who consume diets rich in fruit and vegetables and a lower incidence of cancer (17). Several nutrients found in these foods have shown cancer preventive properties by inhibiting mutagenesis and cell proliferation, as well as by inducing apoptosis (reviewed in (3)). There is evidence of chemopreventive properties from
indole-3-carbinol derivatives in cruciferous vegetables, organosulfur compounds from
garlic, isoflavones from soy such as genistein, and polyphenolics from green tea
(reviewed in (18)). Polyphenolics present in fruits and vegetables are major contributors
to their antioxidant properties. The polyphenolic group can be divided into
hydroxybenzoic acids (e.g. gallic and ellagic acids), hydroxycinnamic acids (e.g. ferrulic
and cafeic acids), flavonoids and hydroxystilbenes (e.g. resveratrol) (19). Flavonoids
(Figure 1) can be glycosylated, methylated or they can appear as aglycones. Flavonoids
are derived from a benzo-γ-pyrone structure, with a benzene derivative in position 2
(flavonoids) or 3 (isoflavonoids). The ring attached to the benzene is a γ-pyrone in
flavonols and flavones and a dyhydro-derivative in flavanols and flavanones. Flavonols
and flavones differ in the C₂-C₃ double bond and in the hydroxyl group in position 3.
Anthocyanidines are compounds chemically and biologically related to flavonoids. They
present an open C ring (20). Flavanols are present primarily in seeds and stems whereas
flavonols and anthocyanidines are located in the skin of fruits (19). There is evidence in
the literature of flavonoid content in different cereal grains, such as wheat, barley, and
rice ((21), (22), reviewed in (23)). Flavonoids from barley, such as (+) catechin, (-)
epicatechin and polymers of (+) catechin and (+) gallocatechin, showed antioxidant
activity in vitro ((22)). There is also evidence of flavonoid content in legumes, such as
soybeans, green and yellow beans. Condensed tannins are phenolic polymers that can
also be found in grains and legumes (reviewed in (24)).
Figure 1. Major types of flavonoids and related structures (20).
Detoxification enzymes and cancer chemoprevention:

Tumor initiation can result from DNA damage by electrophilic compounds or by free radicals generated from the metabolism of carcinogens. In response to this damage, animal cells have evolved mechanisms for protection against toxic compounds (25). One of the principal mechanisms for cellular protection is the elevation of certain Phase II enzymes, such as glutathione-S-transferase (GST), NAD(P)H: quinone reductase (NQO1) and UDP-glucuronosyltransferases (UDP-GT) as well as elevated levels of glutathione (25).

Biotransformation enzymes can be divided into two groups, Phase I and Phase II. Phase I enzymes include the cytochrome P<sub>450</sub> group, cyclooxygenases, and monoamine reductases. Examples of Phase II detoxification enzymes include the quinone reductases, UDP-GTs, and GSTs. Phase I enzymes are involved in reactions that generate electrophilic species. On the contrary, Phase II enzymes either inhibit the formation of electrophiles or catalyze their conversion to inactive and more water-soluble conjugates. This enables them to be rapidly excreted from the body. The balance between Phase I and Phase II enzymes contributes to the risk of chemically induced cancer of an individual (reviewed in (26)).

Phase II enzymes can be regulated / stimulated by monofunctional or bifunctional inducers. Monofunctional inducers cause an electrophilic response in the cell that stimulates the transcription of Phase II enzymes. This type of induction involves binding to the activator protein-1 (AP1) site in the antioxidant-responsive element (ARE). Bifunctional inducers bind the aromatic hydrocarbon (Ah) receptor, forming an inducer/Ah receptor complex which binds to the xenobiotic-responsive element (XRE),
stimulating the transcription of both Phase I and Phase II enzymes (26-28). Induction of Phase I enzymes could potentially lead to the activation of some carcinogens. Therefore, the ideal chemopreventive agent is a nontoxic compound that stimulates Phase II enzymes without inducing Phase I enzymes (26).

The flavoprotein NQO1 [NAD(P)H: quinone oxidoreductase; EC 1.6.99.2] or DT-diaphorase is a two-electron quinone reductase that uses NADH or NADPH as reducing cofactors. NQO1 (Figure 2) reduces quinones directly to hydroquinones. This bypasses potentially toxic semiquinones, avoiding the formation of free radicals generated in redox cycling. Hydroquinones can then be conjugated and excreted from the body (27, 29, 30).

![Figure 2. Quinone metabolism by NQO1 (27).](image)

NQO1 is a homodimeric flavoprotein, containing one FAD molecule in each subunit. Crystal structure studies reveal the existence of two polypeptide chains that dimerize to form two identical catalytic sites (reviewed in (31)). These observations
support a ping-pong mechanism for the reduction of xenobiotics such as quinones by NQO1. In this reaction, a hydride is transferred from NAD(P)H to FAD and from FADH$_2$ to the quinone substrate. This explains the mandatory two-electron reduction of the quinones by NQO1 (reviewed in (32)). In humans, NQO1 is mainly distributed among tissues that need high antioxidant protection. These tissues include the vascular endothelium, epithelial cells of the breast, lung and colon, adipocytes, epithelium of cornea, lens and retina, and optic nerve (reviewed in (31)). At the cellular level, 90% of the enzyme is found in the cytosol and in smaller amounts associated with the mitochondria and microsomes (reviewed in (29)).

Induction of Phase II detoxification enzymes by natural or synthetic compounds is a well-established strategy towards cancer chemoprevention (25, 26, 33-35). It has been shown that NQO1 induction protects against toxicity, mutagenesis or carcinogenesis (33, 35-37). A wide variety of chemically diverse compounds such as phenolic antioxidants, polycyclic aromatic hydrocarbons, azo dyes, flavonoids, coumarin and certain sulfur compounds induced NQO1 in Hepa1c1c7 murine hepatoma cells (38) and showed chemoprotective effects \textit{in vivo} (7). Certain antioxidants such as BHA [2(3)-tert-butyl-4-hydroxyanisole], BHT (3,5-di-tert-butyl-4-hydroxytoluene), ethoxyquin (1,2-dihydro-6-ethoxy-2, 2,4-trimethylquinoline), and disulfiram [bis(diethylthiocarbamyl)disulfide] stimulated NQO1 and protected against polycyclic aromatic hydrocarbon-induced cancer (reviewed in (36)). The protective effects of these antioxidants were associated with their ability to stimulate NQO1 and other detoxification enzymes. Induction of such enzymes results in the inactivation of electrophiles, and as a consequence, in protection against free radical damage (36, 39-41).
Diets rich in fruits, vegetables, whole grains and legumes have been associated with a decreased cancer risk. High phytoestrogens concentrations contained in those diets are believed to be in part responsible for the protective effect. Dietary phytoestrogens such as genistein, enterolactone, biochanin A and coumestrol increased NQO1 expression in colonic Colo205 cells after a 48-hour exposure to different concentrations (0.001 to 10.0 μM) of the compounds. The highest increase in the expression (6 to 8-fold) was observed with enterolactone and genistein. These two compounds doubled NQO1 activity at concentrations of 0.14 and 0.04 μM, respectively, followed by biochanin A (1.1 μM) and coumestrol (12.0 μM) (42). There is also evidence of the correlation between the ability of coumarin and 4’-bromoflavone to induce NQO1 and their protection against chemically induced cancer (43, 44). Quercetin is one of the most prevalent flavonoids in the diet. It has cytoprotective and anti-tumorigenic properties as shown in different animal studies, where quercetin inhibited colon, skin and mammary tumors (45).

Exposure of quercetin (15 μM) to a human breast carcinoma cell line (MCF-7) for a period of 24 h resulted in a two-fold increase in NQO1 activity and a three- to four-fold increase in NQO1 gene expression (46).

Flavonoids from onions induced NQO1 after a 24-hour exposure in Hepa1c1c7 cells. Quercetin aglycone was the best NQO1 inducer, followed by quercetin-4’-glucoside. The 3’glycosylation suppressed their ability to induce NQO1 (47). Unfortunately, Hollman et al. showed that flavonoid glycosides are better absorbed from the diet than the aglycone form (48).

A correlation between NQO1 expression and prevention against benzo(a)pyrene (BP) induced cancers (foregutstomach and lung) was observed in mice fed with diets
containing 0.25 μmol of garlic organosulfides. Diallyl disulfide (DADS) and diallyl trisulfide (DATS) caused a 2.4 and 1.5-fold increase in forestomach NQO1 activity and were potent inhibitors of BP induced forestomach cancer. Diallyl sulfide’s (DAS) low induction of NQO1 also correlated with a low protective effect against BP induced forestomach carcinogenesis. In the same manner DAS was a potent inhibitor of lung cancer and caused a 3.2-fold induction in NQO1 activity in the lung. DATS caused a 1.5-fold induction of NQO1 activity in the lung and did not inhibit BP-induced lung cancer (49).

Resveratrol, a polyphenolic present in grapes, showed cancer chemoprotective activity by inhibiting carcinogenesis at the initiation, promotion and progression steps (50). The anti-initiation protection is associated with the induction of Phase II detoxification enzymes. Prochaska et al. (28) showed that resveratrol doubled NQO1 activity in Hepa1c1c7 cells at a concentration of 21 μM.

Flavonoids from hops (2', 4', 6', 4-OH-3-prenylchalcone and isoxanthohumol) also inhibited activation of aflatoxin B1 by cytochrome P450 (51), and induced NQO1 in Hepa1c1c7 cells, without stimulating CYP1A1. Hop chalcones (xanthohumol and dehydrocycloxaanthohumol) also induced NQO1 in a mutant cell line deficient in Ah-receptor (Hepa 1c1c7 bp' cl) (52).

Diets rich in vegetables, especially those high in cruciferous vegetables, such as cabbage, cauliflower, broccoli and Brussel sprouts are correlated with lower cancer incidences (53). Cruciferous vegetables are rich in isothiocyanates, dithiolthiones and indole-3-carbinol (I3C) derivatives (54). Sulforaphane, an isothiocyanate present in broccoli induced NQO1 in Hepa1c1c7 cells. It also stimulated GST and NQO1 in
different mouse tissues (53, 55, 56). Sulforaphane and its glucosinolate, glucoraphanin, inhibited the incidence and number of dimethylbenz(a)anthracene-induced mammary tumors in rats (56). Glucobrassicin, an I3C derivative found in high concentrations in cruciferous vegetables reduced the multiplicity and incidence of mammary tumors in mouse and rats (54, 57). I3C and related compounds can also stimulate detoxification enzymes, such as NQO1 and GST (reviewed in (18)).

There is evidence of polyphenolic content in different cereal grains and legumes, such as wheat, barley, rice, soybeans and lentils (21, 22) (24). Rice polyphenolics appeared to play a protective role in oxidative stress (23). Flavonoids from cereal grains, such as wheat and barley, have shown antioxidant properties in vitro (21, 22). As mentioned earlier, some antioxidants have cancer protective effects due to their ability to stimulate detoxification enzymes, such as NQO1 (reviewed in (36)). Therefore, flavonoids and other polyphenolics from cereal grains and legumes may prevent cancer, by stimulating detoxification enzymes such as NQO1.
Cyclooxygenase-2 and cancer chemoprevention:

Prostaglandin endoperoxide H synthase (PGHS), also known as cyclooxygenase (COX) is involved in the synthesis of prostaglandins (PGs) and other prostanoids from arachidonic acid (AA). AA is a 20-carbon polyunsaturated fatty acid that exists as an ester of membrane phospholipids. Activation (Figure 3) of phospholipase A₂ by chemical or mechanical stimuli results in the hydrolysis of the ester bond in membrane phospholipids and the release of free arachidonate (reviewed in (58, 59)). Subsequently, PGHS catalyzes the conversion of arachidonate and O₂ to PGG₂, an unstable compound that rapidly undergoes a two-electron reduction to PGH₂.

Figure 3. Prostanoid biosynthetic pathway (58).
In the cyclooxygenase reaction, an arachidonyl radical is formed by abstraction of the (13S)-hydrogen, followed by oxygen additions at C-11 and C-15 to generate PGG2. Then, the peroxidase activity of the enzyme reduces the hydroperoxide in position 15 to an alcohol, resulting in PGH2 formation (60). PGH2 is the precursor for all prostanoids, such as prostaglandins, prostacyclin, and thromboxanes. These compounds bind to specific G-protein-linked receptors and alter second messenger levels (58).

There are two known isozymes of COX: COX-1 and COX-2. Both isozymes are very similar structurally, with a 60% homology in amino acid sequence (58, 61). They are both homodimeric, heme-containing proteins with two catalytic sites (62). They also have similar cyclooxygenase turnover rates (~3500 mol of arachidonate/min/mol of dimer) and $K_m$ values for arachidonate (~5 μM). The residues involved in the catalytic site are conserved in both isozymes (reviewed in (58)). Despite these similarities, COX-1 and COX-2 differ in some aspects, such as their expression patterns and their distribution. COX-1 is expressed constitutively in platelets, kidney, colon, stomach and most tissues, and it is related to the normal physiological functions of prostaglandins, such as cytoprotection of the gastric mucosa, regulation of renal blood flow and platelet aggregation (reviewed in (63)). In contrast, COX-2 levels are undetectable in most normal tissues, but are rapidly expressed in response to cytokines, oncogenes, tumor promoters and growth factors. Although COX-2 is considered the inducible isozyme, COX-2 is expressed constitutively in kidney and parts of the brain (61).

The genes for COX-1 and COX-2 are located in human chromosome 9q32-q33.3 and 1q25-q25.3 respectively (reviewed in (64)). There are significant differences between the COX isozymes in gene structure, mRNA stability and promoter regions. COX-2
contains in its 3′-untranslated region multiple copies of Shaw-Kamen sequences (AUUUA), which are responsible for the instability of COX-2 mRNA. This instability is characteristic of an early response gene. COX-2 also presents a TATA box in its 5′-flanking region, which is absent in COX-1, a classic housekeeping gene (65). CCAAT/enhancer-binding protein, and cyclic AMP-responsive element are regulatory elements present in the 5′-flanking region. There are also an activator binding protein, SP1 and NF-Kappa B sites (reviewed in (64)).

There are also some structural differences between COX-1 and COX-2. COX-2 contains an additional 18-amino acid sequence close to the C terminus that is absent in COX-1 (58, 61). The hydrophobic channel that conducts to the active site of COX-2 also has an additional pocket and a slightly larger orifice than COX-1. This is the result of the substitution of an isoleucine in COX-1 for a valine in COX-2. Drugs that bind to this additional pocket are strong selective COX-2 inhibitors (66). They also differ in their pattern of migration on SDS-PAGE: COX-1 moves as a single band with a relative molecular weight of 72 kDa while COX-2 migrates as two bands at 72 and 74 kDa. The 72 kDa band in COX-2 has three linked oligosaccharides while the 74 band contains four linked oligosaccharides. Both COX-1 and COX-2 are glycosylated proteins, as N-glycosylation is essential for the folding of the enzymes into their active conformation.

The biosynthesis of prostaglandins can follow two kinetically different responses, the immediate and the delayed response. The immediate prostaglandin synthesis takes place minutes after the cell is exposed to a pro-inflammatory stimulus. Such stimuli result in increased Ca\(^{2+}\) levels, activation of cytosolic phospholipase A\(_2\) and AA release, which is metabolized mainly by COX-1. The delayed synthesis of prostaglandins occurs over a
long period of time, and involves the induction of COX-2. The amount of AA available
determines the COX isozyme participating in the synthesis of prostaglandins. The
involvement of COX-2 in the inflammatory delayed response is due to its ability to
metabolize lower concentrations of AA than COX-1 (reviewed in (67)).

COX-1 (Figure 4) is part of a prostanoid biosynthetic pathway located in the
endoplasmic reticulum (ER). PGH₂ synthesized by COX-1 in the lumenal part of the ER
migrates to the cytosolic side where it is converted to different prostanoids.

Figure 4. Model for subcellular distribution of PGHS-1 and -2 prostanoid
biosynthetic pathways. Abbreviations: C, carrier; E, effector; G, G protein; R, receptor;
TXA-S, thromboxane synthase; sPLA₂, secretory PLA₂; cPLA₂, cytosolic PLA₂; PGI-S,
prostacyclin synthase; and Rn, nuclear receptor (58).
Prostanoids derived from this pathway migrate then to the cell surface where they bind to specific G-protein linked receptors. COX-2 is part of the nuclear prostanoid synthetic pathway. Products of this pathway act at the nuclear level and they may play a role in cell differentiation and proliferation (58). COX-1 derived products are involved in the normal functions of prostaglandins, such as cytoprotection of the gastric mucosa and regulation of renal blood flow. The COX-2 derived prostanoids are primarily involved in inflammation. This classification led to the development of COX-2 specific inhibitors. The new class of drugs would theoretically be able to block inflammation without altering the regulatory functions of the prostaglandins therefore, avoiding the adverse effects associated with the traditional non-steroidal antinflammatory drugs (NSAIDs). This view subsequently resulted in an oversimplification. COX-1 was also shown to have a role in the inflammatory process and several additional functions of COX-2 were also discovered. COX-2 is involved in the development of kidneys and maintenance of renal function, protection of the gastrointestinal tract from infection, ovulation and uterine implantation (reviewed in (63)). Observations from COX-1 and COX-2 knockout mice also provided additional information about their specific functions. COX-1 deficient mice suffered difficulty in parturition and impaired platelet aggregation, while COX-2 deficient mice were infertile and presented kidney malfunction (68). There is evidence for the involvement of COX-2 in cancer. Elevated levels of prostaglandins in intestinal adenomas and colorectal cancers were associated with over-expression of COX-2, but not COX-1 (69). COX-2 is highly expressed in several tumors such as colorectal carcinoma (70), squamous cell carcinoma of the esophagus (71), gastric (72), pancreatic (73), and lung cancer (74-76).
Epidemiological studies have shown that aspirin and other NSAIDs reduce the risk of colorectal cancer by 40% to 50% (77-80). Also, studies conducted by Sheng et al. (81) showed that a selective COX-2 inhibitor (SC-58125) reduced the tumor formation in nude mice after implantation of HCA-7 human colon cancer cells by 85 to 90%. Implants of HCT-116, lacking COX-2 expression were not affected by the administration of the COX-2 inhibitor (81). Oshima et al. (82) studied the role of COX-2 in tumorigenesis using Apc$^{\Delta716}$ knockout mouse, a model for human familial adenomatous polyposis (FAP). A reduction in the number and size of intestinal polyps was observed when the gene encoding for COX-2 was disrupted. The response was gene dosage-dependent, with an 86% reduction in the number of polyps in COX-2 null mice vs. a 66% decrease when only one copy of the gene was knocked out. There was also a dose-dependent reduction in the number of polyps in mice treated with a COX-2 selective inhibitor (82).

The formation of new blood vessels or angiogenesis is an essential process for the growth of tumors beyond 2-3 mm (83). Tumors transplanted to angiogenic-deprived conditions remained permanently dormant. After blood supply was restored, a rapid tumor growth was observed (84). One promising target in cancer research is the inhibition of blood vessel formation by angiogenesis inhibitors. The rationale for the design of these drugs is that by blocking the formation of blood vessels, the oxygen and nutrient supply to the tumor is cutoff and the capacity of the tumor to grow and spread to other parts of the body is reduced. The formation of new blood vessels is the result of the balance between stimulatory and inhibitory growth factors, tumor suppressor genes, oncogenes, proteases, cytokines, signal transduction enzymes and endogenous modulators (85). There are a large number of endothelial cell growth stimulators,
including vascular endothelial growth factor (VEGF), acidic and basic fibroblast growth factor (aFGF and bFGF), platelet-derived growth factor, epidermal growth factor, angiogenin, angiopoietin-1, interleukin-8, among others. Some of the angiogenesis inhibitors include endostatin, angiopoietin-2, tissue inhibitor of metalloproteinase-1 and 2, thrombospondin, interferon-alpha and interleukin-10 and 12 (85, 86). Among the stimulatory factors, VEGF and bFGF are two main targets for antiangiogenic therapy. VEGF and bFGF are up-regulated in many tumors where they regulate the proliferation and migration of endothelial cells (reviewed in (87)). Basic FGF and VEGF stimulate capillary tube formation in a synergistic way (reviewed in (88)).

Angiogenesis is involved in the transformation of pre-malignant lesions to malignant lesions. There is also a correlation between the degree of vascularization of a tumor, its invasiveness and its metastatic potential (reviewed in (85)). The formation of blood vessels allows the tumor to have access to the systemic circulation, so tumor cells can migrate to distant locations and form metastatic foci (89). COX-2 expression in human colon cancer cells was associated with elevated RNA levels of metalloproteinase-2 and increased metastatic potential. Sulindac sulfide, a COX-2 inhibitor, reversed both prostaglandin production and invasiveness (90).

Aspirin and COX-2 specific inhibitor NS-398 inhibited angiogenesis in Caco-2 colon carcinoma cells when cocultured with endothelial cells. These drugs block the formation of new blood vessels by inhibiting COX-2 and decreasing the synthesis of growth factors. COX-2 overexpression in those cells was associated with stimulation of endothelial cell motility and increased tube formation due to the high production of angiogenic factors. COX-2 inhibition reversed those effects, which suggested that the
prostanoids synthesized by the enzyme are involved in the angiogenic process. COX-1 was upregulated in endothelial cells by angiogenic factors, where it may play an important role in angiogenesis. COX-1 inhibition may be of interest in tumors that lack COX-2 expression (83).

Masferrer et. al (91) also showed that the COX-2 inhibitor celecoxib inhibited FGF-induced corneal angiogenesis and suppressed growth in human colon cancer cells (HT-29) and Lewis lung carcinoma cells. The number of metastases observed was reduced at high drug dosages when these cells were implanted in mice (91). Both COX-2 expression and angiogenesis share the Ras/Raf-1/ extra-cellular signal-regulated kinase (ERK) pathway. NSAIDS inhibited ERK2 (MAPK) activity in rat aortic endothelial cells, human dermal microvascular endothelial cells, and human umbilical vein endothelial cells (92).

COX-derived prostaglandin E1 and E2 stimulated the formation of new blood vessels in mouse fibroblasts, an effect that was reversed by indomethacin, a nonselective COX inhibitor (93). Form and Auerbach (94) also showed the angiogenic potential of PGE2 in the chorioallantoic membrane model. PGE2 pellets with releasing rates of 0.2, 2.0, or 20 ng of PGE2/day induced the formation of new blood vessels in the membrane. In contrast, pellets of PGA2, PGF2, or TXB2, with similar releasing rates did not have that effect, suggesting that PGE2 may play a critical role in the neo-vascularization process (94). There is evidence of PGE2 stimulating the expression of VEGF in synovial fibroblasts (95) and in a non-neoplastic pre-osteoblastic cell line (96). VEGF and bFGF transcription were also stimulated by PGE2 in a retinal glial cell line (97).
Flavonoids are benzo-γ-pyrone derivatives that are widespread in nature. Preparations containing flavonoids have been used empirically in the past to treat human diseases (20). Flavonoids are also ingested in biologically significant concentrations as part of certain diets (98). There is evidence in the literature of flavonoid content in different cereal grains, such as wheat and barley (21, 22). Flavonoids from barley, such as (+) catechin, (-) epicatechin and polymers of (+) catechin and (+) gallocatechin, showed antioxidant activity in vitro (22). Some antioxidants, such as pyrrolidinedithiocarbamate and N-acetylcysteine decrease COX-2 expression, PG synthesis, and proliferation in colorectal cancer cells (99).

Diet rich in cereal grains inhibited the activity and expression of COX-2 and inducible nitric oxide synthase (iNOS) in colon tumors. They also reduced the incidence, size and multiplicity of azoxymethane-induced colon cancer in rats when compared to animals fed with the non-fortified standard diet (100). Polyphenols from green and black tea, and theaflavins inhibited COX-dependent arachidonic acid metabolism in normal human colon by 37-62 %, at 30ug/ml (101). Possible chemopreventive compounds, such as quercetin, genistein, kaempferol, resveratrol and resorcinol, sharing a resorcin-type structure, suppressed COX-2 transcription in colon cancer cells (102). Flavonoids, including 3-hydroxyflavone, 3’, 4’-dihydroxyflavone, 2’, 3’-dihydroxyflavone, fisetin, luteolin and apigenin also showed antiangiogenic and antiproliferative properties in vitro. They blocked bFGF- and VEGF-induced angiogenesis (98). Therefore, flavonoids from cereal grains may have the potential to inhibit the carcinogenic process at the promotion and progression step, by inhibiting COX-2.
METHODS DEVELOPMENT

FLAVONOYD EXTRACTION:

Preliminary studies were conducted to improve the method of extraction of flavonoids from cereal grains. In the first extractions, fifty grams of each grain were ground to a fine powder and stirred in an 80% aqueous methanol solution for approximately 48h. Then, the mixture was filtered using a Whatman #1 filter, and the methanol was evaporated in a Yamato RE 200 rotary evaporator. Next, several chloroform extractions were performed to the aqueous fraction to eliminate lipid soluble components (103). The remaining solution was extracted with equal volumes of ethyl acetate (103-105). The ethyl acetate was evaporated to dryness, and the residue was re-dissolved in 1-1.5ml of 100% ethanol approximately, and stored at -20°C until assays were performed. We observed an insoluble residue in the bottom of the vials of several extracts and decided to use the supernatants for both NQO1 and COX-2 assays. We attributed some of the variability of the results to the non-homogeneous nature of the flavonoid extracts. We were also concerned about the possible variability in the concentrations of flavonoids used, and therefore not obtaining consistent results.

In an attempt to obtain more consistent results, we prepared a new set of extractions, using a more standardized procedure. This second time we dissolved the residue obtained after evaporation of the ethyl acetate fraction in 5 ml of 100% ethanol, and we filtered the extracts to eliminate the insoluble residue. We also performed a quantification assay to measure the concentration of total phenolics present in the extracts. In some extracts, we observed that the aqueous fraction was more colorful than
the ethyl acetate fraction, suggesting that flavonoid glycosides may be removed in the aqueous fraction.

**COX-2 IMMUNOBLOTTING:**

Preliminary studies were performed with the first set of extracts. In these experiments, U87 cells were exposed for 48 h to 5 to 100-fold dilutions of the extracts, depending on toxicity. The purpose of the preliminary studies was to optimize the dilutions of primary and secondary antibodies utilized, amount of protein loaded, time of blot incubation with the ECL+ detection system, and time of film exposure. For optimal results, 10 μg of protein was loaded for each sample, subjected to electrophoresis for 40 minutes at 200V, and subsequently transferred to a PVDF membrane for 1h at 90V. Incubation with a 1:500 dilution of COX-2 rabbit polyclonal antibody, and a 1:1000 dilution of horseradish peroxidase (HRP) goat anti-rabbit antibody, resulted in optimal visualization of the COX-2 bands at 72 and 74 kDa. Beta-actin was utilized to control protein loading. The membrane was incubated with a 1:10,000 dilution of mouse monoclonal anti-actin antibody for 1h, followed by incubation with a 1:2000 dilution of goat anti-mouse secondary antibody for 1h. The actin bands were visualized at an apparent molecular weight of 42 kDa. A lysate of lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophage cells was used as a COX-2 positive control. There is evidence in the literature of the LPS stimulation of both COX-2 mRNA and protein expression (106, 107).

In our first attempt to quantify the potential COX-2 inhibition, we used the ECL chemiluminescence system and the NIH Image 1.60 program to quantify the bands. The
ECL detection system has been designed for detection of immobilized proteins linked to a HRP antibody. Exposure of the HRP-linked antibody to the ECL reagent in alkaline conditions results in the oxidation of luminol, a cyclic diacylhydrazide. Following oxidation, luminol reaches an excited state, from which it decays to a ground state, emitting light at a maximal wavelength of 428 nm. The light emitted by the enhanced chemiluminescence reaction is maximal after 5-20 minutes, and can be captured in an autoradiography film, such as Hyperfilm ECL (108). Protein quantification by densitometry is possible due to the linear response of light emission with enhanced chemiluminescence. A ratio between the density of the COX-2 bands and the actin bands was calculated to control irregular protein loading. With this method we encountered several problems, such as the inability to quantify overlapping bands and the inaccuracy of the measurements. We decided to use a phosphoimager to quantify the bands after incubation with the ECL Plus enhanced chemiluminescence reagent. The ECL Plus system is based in the oxidation of Lumigen PS-3 acridan substrate by HRP and peroxide. The reaction generates an acridinium ester, which in the presence of peroxide in alkaline conditions produces an excited product that produces a longer and more intense light emission than the standard ECL system (109). A 3-4 minute exposure to 2 ml of the ECL Plus reagent resulted in the best band detection. The maximal light emission was detected at 430 nm with a Fuji Film FLA-3000 phosphoimager. The Image Gauge software was used to quantify the bands.

Although Western blotting is considered a qualitative method for protein expression, we attempted to quantify the COX-2 expression to study the hypothetical relationship between COX-2 inhibition and polyphenolic concentration present in the
grain extracts. High variability was observed in the quantification process. This variability can be attributed to the difficulty of maintaining constant parameters such as efficiency of the transfer, identical antibody dilutions, and equal exposure to the antibodies. We also encountered reproducibility problems in the results. Using virtually the same incubation periods with the polyphenolic extracts, dilutions of the extracts and amount of protein, we observed significant differences between results. To eliminate some variability, U87 cells were serum-deprived for 24 h prior to incubation with the flavonoids. The rationale for the serum starvation was to synchronize cells into the same phase of the cell cycle.

**NQO1 Assay:**

Preliminary studies were conducted with the first set of extracts. Ten-fold and 100-fold dilutions of the extracts in 100% ethanol were used. Experiments were done in triplicate. NQO1 activity was measured in Hepa1c1c7 murine hepatoma cells (29). Briefly, Hepa1c1c7 cells were exposed for 48 h to the flavonoid extracts. NQO1 activity was measured spectrophotometrically at 600nm, using NADH as an electron donor and 2,6-dichlorophenolindophenol (DCPIP) as an electron acceptor. The reduction of DCPIP was used as a measure of NQO1 activity.
MATERIALS AND METHODS

MATERIALS:

Samples of lentils, barley, and wheat were kindly donated by Terry and Taren Grass of Grass and Seed, Inc. of Box Elder, Montana. Other grains were purchased at the Good Food Store in Missoula, Montana. All reagents were at least of analytical grade. All chemicals were purchased from Sigma (St. Louis, MO), except buffers for immunoblotting and protein assay dye reagent (Bio-Rad, Hercules, CA).

CELL CULTURE:

Human glioblastoma (U87) and mouse hepatoma (HepalclcT) cell lines were purchased from the American Type Culture Collection (Rockville, MD). All reagents and media for cell culture were obtained from Life Technologies (Rockville, MD). HepalclcT and U87 cells were grown in minimal essential medium (MEM), supplemented with 10% calf serum and penicillin, streptomycin and glutamine (PSG), at 37°C and 5% CO₂ in a humidified incubator.

FLAVONOID EXTRACTION:

Fifty grams of each grain and the legume, lentils, were ground in a biomixer blender to a fine powder and stirred in an 80% (v/v) aqueous methanol solution for approximately 48h. The mixture was subsequently filtered through a Whatman #1 filter, and the methanol was evaporated in a Yamato RE 200 rotary evaporator. Following methanol evaporation, the aqueous fraction was extracted three times with equal volumes of chloroform to remove the lipid component, as described previously (103). The
remaining solution was extracted with ethyl acetate (103-105). The ethyl acetate was evaporated to dryness, and the residue was subsequently re-dissolved in 5 ml of 100% ethanol, filtered and stored at -20°C until assays were performed.

**TOTAL PHENOLIC ASSAY:**

The Prussian blue method was used for polyphenolic quantification of the grain extracts (described in (110)). When searching for a phenolic quantification method, we looked for a simple assay that would allow us to quantify a wide variety of grain extracts. The most common quantification methods of general phenolics are based on a redox assay, where the oxidation of phenolic groups can be detected spectrophotometrically. Non-phenolic antioxidants present in plants can interfere with these redox reactions. The Prussian blue method has less interference with non-phenolic compounds than other quantification assays, such as the Folin method (reviewed in (111)). The Prussian blue method is based on the reduction of Fe\(^{3+}\) to Fe\(^{2+}\) by polyphenols, and the subsequent formation of a ferricyanidine-ferrous ion complex, also known as Prussian blue. This colored complex can be read spectrophotometrically at 720 nm.

One hundred µl of each extract was added to 50 ml of distilled water in a 125 ml Erlenmeyer flask. To each flask, 3 ml of 0.1 M FeNH\(_4\)(SO\(_4\))\(_2\) in 0.1 M HCl was added at 1 minute intervals, swirling after each addition. Following a 20 minute interval, timed additions of 3 ml of 8 mM K\(_3\)Fe(CN)\(_6\) were started. Solutions were mixed and after a 20-minute incubation period, the absorbance was read at 720 nm using a Beckman DU 7500 spectrophotometer (111). The timing of the additions was essential for obtaining consistent results. A time-dependent increase in the absorbance was observed. A standard
curve was obtained following the same timed process, using (+)-catechin as a standard phenolic. Catechin concentrations of 0 mM, 5mM, 10mM, 15mM and 20mM (in 100% ethanol) were used to build the standard curve. A 100% ethanol blank was used to correct the absorbance.

**NQO1 ACTIVITY ASSAY:**

Hepalc1c7 cells were grown to 50-60% confluency in the conditions described above. Cells were then exposed for 48h to fresh medium containing the flavonoid extracts in ethanol (1%, by volume). A 10-fold dilution of the original extracts was prepared in 100% ethanol. Three hundred µl of the diluted extracts were added to 30 ml of media. Ten ml of media/extract were then pipeted into each petri dish. Following the period of exposure, media was removed and cells were washed with 2 ml of phosphate buffered saline (PBS). Subsequently, cells were trypsinized and centrifuged for 5 min, at 4°C and 2500 r.p.m in a Tomy TX-160 high-speed refrigerated microcentrifuge. The supernatant was removed and cells were washed twice by re-suspending the cells in 4 ml of PBS followed by centrifugation. The pellet was re-suspended in 1 ml of Tris-Sucrose solution, then sonicated and stored at -20°C until the assay was performed. NQO1 activity was measured spectrophotometrically in a reaction mixture containing Tris-HCl 25mM (PH 7.4), 0.7 mg/ml BSA (bovine serum albumin) and, 0.1% Tween 20. NADH was used as an electron donor and DCPIP as an electron acceptor as described previously (29). Twenty µl of NADH was added to the reaction mixture in a 1 ml plastic cuvette, followed by addition of 20 µl of the cell sonicate. The reaction was started by the addition of 20 µl of DCPIP to the reaction mixture containing both NADH and the cell sonicate. The
disappearance of DCPIP was measured at 600nm for 30s in a Beckman DU 7500 spectrophotometer.

NQO1 activity was measured with and without addition of dicoumarol, to calculate the activity due to this enzyme and to eliminate other possible causes of DCPIP reduction (27). Dicoumarol is a potent inhibitor of NQO1, with a Ki of $10^{-8}$ M. It inhibits NQO1 by competing with NADH and NADPH (29). The portion of activity inhibited by dicoumarol was used as a measure of NQO1 activity.

**COX-2 IMMUNOBLOT ANALYSIS:**

U87 cells were grown to 50% confluency in the conditions described above. Cells were then serum deprived for 24 h in order to synchronize them into the same phase of the cell cycle. After the period of serum starvation, cells were fed with normal medium and exposed to the flavonoid extracts at two different concentrations:

-1x dilution of the extracts: 300 µl of full strength extract in 30 ml of medium.
-10x dilution of the extracts: 30 µl of full strength extract + 270 µl of 100% ethanol, in 30 ml of medium. In both cases, 10 ml of media/extracts was added to each petri dish. Three plates were used per sample. Cells were then scraped serially in 750 µl of two-fold concentrated Laemmli buffer [10% SDS, glycerol, 1 M Tris-HCl (PH 6.8), supplemented with protease inhibitors (40 µg/ml PMSF, 2 µg/ml aprotinin and 2 µg/ml leupeptin)]. Samples were passed through an 18 g and a 25 g needle and boiled for five minutes. Samples were stored at -20° C until further use.

Samples containing a total of 10 µg of protein were then added to SDS-PAGE loading buffer [62.5 mM Tris-HCl (PH 6.8), 2% SDS, glycerol, bromophenol blue and
5% beta-2-mercaptoethanol] and heated for 5 min at 100° C. The samples were subsequently loaded onto a 10% polyacrylamide gel and subjected to electrophoresis for 40 minutes, at 200V in a Bio-Rad mini-protean 3 cell (Bio-Rad, Hercules, CA). Gels were subsequently transferred to a PVDF membrane (Osmonics, Westborough, MA) for 1h, at 90 V.

The membranes were blocked with 3% non-fat milk in TBST [100mM Tris Base, 150mM NaCl, 0.1% Tween 20 (pH 8.0)], and subsequently incubated with a 1:500 dilution of COX-2 rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), and a 1:10,000 dilution of mouse monoclonal anti-actin antibody (Oncogene, Boston, MA) for 1 h. Following, a 1:1000 dilution of goat anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA), and a 1:2000 dilution of horseradish peroxidase goat anti-mouse antibody (Oncogene, Boston, MA) were applied. A macrophage cell line, RAW 264.7 was used as a COX-2 positive control. Bands were visualized by ECL Plus enhanced chemiluminescence system (Amersham Pharmacia Biotech, Piscataway, NJ). Protein bands in Western blots were quantified by densitometry, using a Fuji Film FLA-3000 phosphoimager.

**BRADFORD PROTEIN ASSAY:**

Protein concentrations were determined using the Bradford protein assay (112) from Biorad (Hercules, CA). The principle of this assay is the change of color that occurs when protein binds to the Coomassie blue dye present in the Bradford reagent. The Coomassie blue dye binds mainly to aromatic and basic amino acids, especially to arginine, changing the absorbance from 465 nm to 595 nm. The change of color observed is proportional to the protein concentration.
BSA concentrations of 0, 100, 200, 300, 400, and 500 µg/ml were used for a standard curve. Samples and standards were exposed to a 5-fold dilution of the reagent for at least 5 minutes. Absorbance was read in a Beckman DU 7500 spectrophotometer at 595 nm.
RESULTS

Preliminary studies were conducted with a first set of extracts. Briefly, fifty grams of each grain were ground to a fine powder, stirred in 80% aqueous methanol for 48 h, and extracted with chloroform and ethyl acetate, as described in the methods section. The ethyl acetate fraction was evaporated, and the residue was dissolved in 1-1.5 ml of 100% ethanol. We attributed part of the variability of the results observed with the first set of extracts to the non-homogeneous nature of the flavonoid extracts. In order to obtain more consistent results, we prepared a second set of extracts, in which the residue obtained after evaporation of the ethyl acetate fraction was re-dissolved in 5 ml of 100% ethanol, and filtered to eliminate the insoluble residue. The Prussian blue method was used to quantify the polyphenolic content of the second set of extracts.

Polyphenolic quantification of extracts from cereal grains and legumes:

Figures 5, 6, and 7, show the polyphenolic content of the second set of extracts. Dehulled barley and row barley have the highest concentrations of total phenolics, with concentrations of 10.3 ± 0.5 mM and 9.8 ± 0.5 mM, respectively. All types of wheat ranged from 0.20 ± 0.47 x 10^{-2} mM to 0.56 ± 0.02 mM, containing the lowest polyphenolic concentrations of the grains analyzed. A marked difference was observed in the polyphenolic concentrations of brown rice and wild rice. Wild rice, the most unprocessed form, contains a phenolic concentration 5 times higher than brown rice (3.7 ± 0.1 mM vs 0.7 ± 0.1 mM). Oats, rye, lentils and maize have a phenolic content of 1.2 ± 0.04 mM, 0.9 ± 0.05 mM, 3.9 ± 0.3 mM, and 1.5 ± 0.1 mM, respectively.
Effect of flavonoids from cereal grains and legumes on NQO1 activity:

Two different sets of extracts were used to evaluate the effect of flavonoids from cereal grains and legumes on the enzymatic activity of NQO1. Preliminary studies done with the first set of extracts (Tables 1, 2, 3, and Figure 8), showed a significant NQO1 (P<0.05) induction with most extracts. Hepalclc7 cells were exposed for 48 h to 10-fold dilutions of lentils, kamut, spring hard wheat, spring red wheat, winter wheat, dehulled barley, and spelt extracts. The extracts of oats, rye, row barley, maize, wild rice, and brown rice were used at 100-fold dilution to avoid the toxicity observed with more concentrated solutions. In the wheat group, induction rates ranged from a 1.6-fold induction with spring red wheat to a 3.9-fold induction with spelt. The induction observed with spelt polyphenols is considerable when compared to the NQO1 induction observed with standard phenolic inducers, such as tert-Butylhydroquinone (t-BHQ) and hydroquinone (HQ), used as positive controls. Exposure of t-BHQ (20 μM) and HQ (20 μM) (Table 3) to Hepalclc7 cells for 48 h resulted in a 3.6, and 3.2-fold NQO1 induction, respectively.

Row barley polyphenols caused a 2.6-fold induction in the enzyme’s activity, whereas dehulled barley caused a 1.4-fold induction. Incubation with wild rice polyphenols caused a 2.7-fold induction, but only a 1.3-fold induction was observed when cells were exposed to brown rice polyphenols. A 2.5, 2.4, 2.9, and 1.4-fold induction of NQO1 activity was observed with the extracts of lentils, oats, rye and maize, respectively. The NQO1 induction observed with maize and brown rice was not significant. When experiments were repeated with the second set of extracts (Table 4, 5, and Figure 9), only the extracts of spring red wheat, winter wheat, wild rice, and lentils
caused a significant NQO1 induction. A ten-fold dilution of the second set of extracts was exposed to Hepa1c1c7 cells for 48h. A 1.5 (P<0.05), 1.6 (P<0.01), 1.5 (P<0.05) and 1.8-fold (P<0.01) NQO1 induction was observed with the flavonoids of spring red wheat, winter wheat, wild rice, and lentils, respectively. No correlation was observed between the amount of polyphenolics in the extracts and the NQO1 induction.

**Effect of flavonoids from cereal grains and legumes on the inhibition of COX-2 protein expression:**

Preliminary studies conducted with the first set of extracts showed COX-2 inhibition with oats and rye polyphenols (Figure 10), suggesting their potential cancer preventive properties. In these experiments, U87 cells were exposed to a 5 to 10-fold dilution of the extracts for 48 h. Further experiments were conducted with the second set of extracts to evaluate these properties.

A 10-fold dilution of the extracts and the undiluted extracts were used to investigate the inhibition of COX-2 expression in U87 cells. No significant COX-2 inhibition was observed after a 48-h exposure to a 10-fold dilution of the extracts or to the extracts at full strength. COX-2 Western blots of U87 cells incubated with a 10-fold dilution of the extracts are shown in Figures 11 and 12. Figures 13 and 14 show the COX-2 immunoblots of cells incubated with the polyphenolic extracts at full strength. Oats and dehulled barley extracts were toxic to U87 cells when used at full strength. Both extracts caused the de-attachment of the cells from the bottom of the plates.

The quantification of the bands by densitometry (Tables 6, 7, 8 and 9) confirmed the results observed in the Western blots. The density of the actin bands was used to correct differences in protein loading.
Figure 5. Polyphenolic quantification of the various grain and legume extracts, using the Prussian blue method. The extracts were prepared from 50 g of each grain and legume. The formation of the ferricyanidine-ferrous ion complex was read spectrophotometrically at 720 nm. Bars represents the means ± S.D. (n=3).
Figure 6. Polyphenolic content of brown rice, and wild rice, using the Prussian blue method. The extracts were prepared from 50 g of each grain. The formation of the ferricyanidine-ferrous ion complex was read spectrophotometrically at 720 nm. Bars represents the means ± S.D. (n=3).
**Figure 7.** Polyphenolic quantification of wheat extracts, using the Prussian blue method. The extracts were prepared from 50 g of each grain. The formation of the ferricyanidine-ferrous ion complex was read spectrophotometrically at 720 nm. Bars represent the means ± S.D. (n=3).
Table 1. Effect of flavonoids from the first set of extracts on NQO1 activity in HepalclcT cells after 48 h of treatment:

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Protein (mg/ml)</th>
<th>NQO1 Activity (µmol/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol (1%)</td>
<td>1.211 ± 0.097</td>
<td>0.088 ± 0.017</td>
</tr>
<tr>
<td>Lentils (0.1x)</td>
<td>1.060 ± 0.087</td>
<td>** 0.224 ± 0.007</td>
</tr>
<tr>
<td>Ethanol (1%)</td>
<td>1.361 ± 0.081</td>
<td>0.071 ± 0.006</td>
</tr>
<tr>
<td>Dehulled barley (0.1x)</td>
<td>1.375 ± 0.080</td>
<td>** 0.100 ± 0.004</td>
</tr>
<tr>
<td>Ethanol (1%)</td>
<td>1.528 ± 0.081</td>
<td>0.053 ± 0.002</td>
</tr>
<tr>
<td>Spring red wheat (0.1x)</td>
<td>1.277 ± 0.283</td>
<td>* 0.086 ± 0.010</td>
</tr>
<tr>
<td>Winter wheat (0.1x)</td>
<td>1.481 ± 0.139</td>
<td>** 0.109 ± 0.003</td>
</tr>
<tr>
<td>Ethanol (1%)</td>
<td>1.296 ± 0.077</td>
<td>0.027 ± 0.003</td>
</tr>
<tr>
<td>Oats (0.01x)</td>
<td>1.378 ± 0.085</td>
<td>* 0.066 ± 0.015</td>
</tr>
<tr>
<td>Row barley (0.01x)</td>
<td>1.400 ± 0.018</td>
<td>** 0.072 ± 0.003</td>
</tr>
<tr>
<td>Rye (0.01x)</td>
<td>1.367 ± 0.230</td>
<td>** 0.080 ± 0.004</td>
</tr>
</tbody>
</table>

* Values are the means ± S.D. (n=3).
* = The NQO1 activity is significantly different than that observed with the control (P<0.05).
** = P<0.01.
Table 2. Effect of flavonoids from the first set of extracts on NQO1 activity in Hepa1c1c7 cells after 48 h of treatment:

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Protein (mg/ml)</th>
<th>NQO1 Activity (μmol/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol (1%)</td>
<td>1.161 ± 0.152</td>
<td>0.069 ± 0.010</td>
</tr>
<tr>
<td>Kamut (0.1x)</td>
<td>1.148 ± 0.199</td>
<td>** 0.176 ± 0.023</td>
</tr>
<tr>
<td>Spring hard wheat (0.1x)</td>
<td>1.335 ± 0.173</td>
<td>** 0.161 ± 0.003</td>
</tr>
<tr>
<td>Spelt (0.1x)</td>
<td>0.832 ± 0.021</td>
<td>** 0.267 ± 0.007</td>
</tr>
<tr>
<td>Ethanol (1%)</td>
<td>1.212 ± 0.026</td>
<td>0.024 ± 0.013</td>
</tr>
<tr>
<td>Maize (0.01x)</td>
<td>1.568 ± 0.071</td>
<td>0.034 ± 0.006</td>
</tr>
<tr>
<td>Wild rice (0.01x)</td>
<td>1.673 ± 0.172</td>
<td>* 0.066 ± 0.005</td>
</tr>
<tr>
<td>Ethanol (1%)</td>
<td>0.977 ± 0.060</td>
<td>0.116 ± 0.027</td>
</tr>
<tr>
<td>Brown rice (0.01x)</td>
<td>0.887 ± 0.047</td>
<td>0.153 ± 0.016</td>
</tr>
</tbody>
</table>

*Values are the means ± S.D. (n=3).

* = The NQO1 activity is significantly different than that observed with the control (P<0.05).

** = P<0.01.
Table 3. Effect of hydroquinone (HQ) and tert-butyl hydroquinone (t-BHQ) on NQO1 activity in Hepa1c1c7 cells after 48 h of treatment:

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Protein (mg/ml)</th>
<th>NQO1 Activity (μmol/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol (1%)</td>
<td>0.601 ± 0.306</td>
<td>0.181 ± 0.053</td>
</tr>
<tr>
<td>t-BHQ (20 μM)</td>
<td>0.604 ± 0.321</td>
<td>** 0.646 ± 0.274</td>
</tr>
<tr>
<td>Ethanol (1%)</td>
<td>0.330 ± 0.036</td>
<td>0.177 ± 0.040</td>
</tr>
<tr>
<td>HQ (20 μM)</td>
<td>0.319 ± 0.015</td>
<td>** 0.575 ± 0.088</td>
</tr>
</tbody>
</table>

*Values are the means ± S.D. of at least three experiments (n=3-6).** = The NQO1 activity is significantly different than that observed with the control (P<0.01).
Figure 8. NQO1 induction in Hepa1c1c7 cells after exposure to the first set of flavonoid extracts for 48 h. Ten-fold and 100-fold dilution of the extracts were used, depending on toxicity. Each bar represents the ratio between the NQO1 activity observed with a sample, and the enzyme's activity observed with a 1% ethanol control. Each data point represents the mean ± S.D. (n=3). * = The enzyme's activity is significantly increased compared to control (P<0.05). ** = P<0.01.
Table 4. Effect of flavonoids from the second set of extracts on NQO1 activity in Hepalclc1c7 cells after 48 h of treatment a:

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Protein (mg/ml)</th>
<th>NQO1 Activity (μmol/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol (1%)</td>
<td>0.970 ± 0.564</td>
<td>0.096 ± 0.041</td>
</tr>
<tr>
<td>Kamut (0.1x)</td>
<td>0.859 ± 0.326</td>
<td>0.122 ± 0.048</td>
</tr>
<tr>
<td>Spring hard wheat (0.1x)</td>
<td>0.896 ± 0.563</td>
<td>0.082 ± 0.013</td>
</tr>
<tr>
<td>Spelt (0.1x)</td>
<td>1.089 ± 0.491</td>
<td>0.102 ± 0.029</td>
</tr>
<tr>
<td>Ethanol (1%)</td>
<td>0.663 ± 0.130</td>
<td>0.111 ± 0.029</td>
</tr>
<tr>
<td>Maize (0.1x)</td>
<td>0.885 ± 0.306</td>
<td>0.146 ± 0.031</td>
</tr>
<tr>
<td>Wild rice (0.1x)</td>
<td>0.763 ± 0.207</td>
<td>*0.164 ± 0.044</td>
</tr>
<tr>
<td>Ethanol (1%)</td>
<td>0.696 ± 0.084</td>
<td>0.106 ± 0.084</td>
</tr>
<tr>
<td>Oats (0.1x)</td>
<td>0.784 ± 0.130</td>
<td>0.129 ± 0.013</td>
</tr>
<tr>
<td>Dehulled barley (0.1x)</td>
<td>0.683 ± 0.250</td>
<td>0.137 ± 0.027</td>
</tr>
</tbody>
</table>

a Values represent the means ± S.D. (n=6).
* = The NQO1 activity is significantly different than that observed with the control (P<0.05).
Table 5. Effect of flavonoids from the second set of extracts on NQO1 activity in Hepa1c1c7 cells after 48 h of treatment a:

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Protein (mg/ml)</th>
<th>NQO1 Activity (µmol/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol (1%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Row barley (0.1x)</td>
<td>1.062 ± 0.434</td>
<td>0.077 ± 0.022</td>
</tr>
<tr>
<td>Brown rice (0.1x)</td>
<td>0.968 ± 0.321</td>
<td>0.091 ± 0.032</td>
</tr>
<tr>
<td></td>
<td>1.163 ± 0.334</td>
<td>0.097 ± 0.061</td>
</tr>
<tr>
<td>Ethanol (1%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spring red wheat (0.1x)</td>
<td>1.130 ± 0.338</td>
<td>0.056 ± 0.011</td>
</tr>
<tr>
<td>Winter wheat (0.1x)</td>
<td>1.126 ± 0.368</td>
<td>* 0.087 ± 0.026</td>
</tr>
<tr>
<td></td>
<td>1.155 ± 0.143</td>
<td>**0.092 ± 0.021</td>
</tr>
<tr>
<td>Ethanol (1%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lentils (0.1x)</td>
<td>0.823 ± 0.096</td>
<td>0.071 ± 0.028</td>
</tr>
<tr>
<td></td>
<td>0.818 ± 0.167</td>
<td>**0.125 ± 0.022</td>
</tr>
<tr>
<td>Ethanol (1%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rye (0.1x)</td>
<td>0.604 ± 0.047</td>
<td>0.125 ± 0.025</td>
</tr>
<tr>
<td></td>
<td>0.624 ± 0.057</td>
<td>0.117 ± 0.015</td>
</tr>
</tbody>
</table>

a Values represent the means ± S.D. (n=6).

* = The NQO1 activity is significantly different than that observed with the control (P<0.05).

** = P<0.01.
Figure 9. NQO1 induction in Hepa1c1c7 cells after exposure to a ten-fold dilution of the second set of flavonoid extracts for 48 h. Each bar represents the ratio between the NQO1 activity observed with a sample, and the enzyme’s activity observed with a 1% ethanol control. Each data point represents the mean ± S.D. (n=6). * = The enzyme’s activity is significantly increased compared to control (P<0.05). ** = P<0.01.
Figure 10. COX-2 immunoblot preliminary studies. U87 human glioblastoma cells were exposed to a 5-fold dilution of the first set of extracts. A total of 25 µg of protein of each sample was loaded. RAW 264.7, a macrophage cell line, was used as a positive control. Bands at 72 kDa correspond to COX-2. Expression of β-actin (42 kDa) was used as a control for gel loading. One of three independent experiments is shown (n=3).
Figure 11. Immunoblot analysis of COX-2 in U87 human glioblastoma cells, following a 48 h exposure to a 10-fold dilution of the different extracts. A total of 10 μg of protein was loaded for each sample. RAW 264.7, a macrophage cell line, was used as a positive control. Bands at 72 kDa and 74 kDa correspond to two different isoforms of COX-2. Expression of β-actin (42 kDa) was used as a control for gel loading. One of three independent experiments is shown (n=3-5).
Figure 12. Immunoblot analysis of COX-2 in U87 human glioblastoma cells, following a 48 h exposure to a 10-fold dilution of the different extracts. A total of 10 μg of protein was loaded for each sample. RAW 264.7, a macrophage cell line, was used as a positive control. Bands at 72 kDa and 74 kDa correspond to two different isoforms of COX-2. Expression of β-actin (42 kDa) was used as a control for gel loading. One of three independent experiments is shown (n=3-5).
Table 6. COX-2 expression in U87 cells exposed to a 0.1x dilution of the polyphenolic extracts:

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Ratio COX-2 / β-actin expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol (1%)</td>
<td>0.720 ± 0.222</td>
</tr>
<tr>
<td>Winter wheat (0.1x)</td>
<td>0.677 ± 0.257</td>
</tr>
<tr>
<td>Spring red wheat (0.1x)</td>
<td>0.862 ± 0.276</td>
</tr>
<tr>
<td>Row barley (10x)</td>
<td>0.828 ± 0.376</td>
</tr>
<tr>
<td>Lentils (0.1x)</td>
<td>0.882 ± 0.474</td>
</tr>
<tr>
<td>Brown rice (0.1x)</td>
<td>0.901 ± 0.331</td>
</tr>
<tr>
<td>Wild rice (0.1x)</td>
<td>0.903 ± 0.479</td>
</tr>
<tr>
<td>Maize (0.1x)</td>
<td>0.871 ± 0.286</td>
</tr>
</tbody>
</table>

* Values are the means ± S.D. (n=3-5). Quantification was done by densitometry. None of the samples were statistically different than control.
Table 7. COX-2 expression in U87 cells exposed to a 0.1x dilution of the polyphenolic extracts:

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Ratio COX-2 / β-actin expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol (1%)</td>
<td>0.733 ± 0.256</td>
</tr>
<tr>
<td>Dehulled barley (0.1x)</td>
<td>0.869 ± 0.395</td>
</tr>
<tr>
<td>Oats (0.1x)</td>
<td>0.837 ± 0.391</td>
</tr>
<tr>
<td>Rye (0.1x)</td>
<td>0.723 ± 0.336</td>
</tr>
<tr>
<td>Spring hard wheat (0.1x)</td>
<td>0.742 ± 0.394</td>
</tr>
<tr>
<td>Kamut (0.1x)</td>
<td>0.670 ± 0.351</td>
</tr>
<tr>
<td>Spelt (0.1x)</td>
<td>0.673 ± 0.386</td>
</tr>
</tbody>
</table>

* Values are the means ± S.D. (n=3-5). Quantification was done by densitometry. None of the samples were statistically different than control.
Figure 13. Immunoblot analysis of COX-2 in U87 human glioblastoma cells, following a 48 h exposure to the different extracts at full strength. A total of 10 μg of protein was loaded for each sample. RAW 264.7, a macrophage cell line, was used as a positive control. Bands at 72 kDa and 74 kDa correspond to two different isoforms of COX-2. Expression of β-actin (42 kDa) was used as a control for gel loading. One of three independent experiments is shown (n=3).
**Figure 14.** Immunoblot analysis of COX-2 in U87 human glioblastoma cells, following a 48 h exposure to the different extracts at full strength. A total of 10 µg of protein was loaded for each sample. RAW 264.7, a macrophage cell line, was used as a positive control. Bands at 72 kDa and 74 kDa correspond to two different isoforms of COX-2. Expression of β-actin (42 kDa) was used as a control for gel loading. One of three independent experiments is shown (n=3).
Table 8. COX-2 expression in U87 cells exposed to the polyphenolic extracts at full strength 

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Ratio COX-2 / β-actin expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol (1%)</td>
<td>0.700 ± 0.343</td>
</tr>
<tr>
<td>Rye (F.S.)</td>
<td>0.615 ± 0.358</td>
</tr>
<tr>
<td>Spring hard wheat (F.S.)</td>
<td>0.430 ± 0.335</td>
</tr>
<tr>
<td>Kamut (F.S.)</td>
<td>0.544 ± 0.278</td>
</tr>
<tr>
<td>Spelt (F.S.)</td>
<td>0.737 ± 0.340</td>
</tr>
</tbody>
</table>

*Values are the means ± S.D. (n=3). Quantification was done by densitometry. None of the samples were statistically different than control. F.S. = full strength.*
Table 9. COX-2 expression in U87 cells exposed to the polyphenolic extracts at full strength *:

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Ratio COX-2 / β-actin expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol (1%)</td>
<td>0.695 ± 0.076</td>
</tr>
<tr>
<td>Winter wheat (F.S.)</td>
<td>0.733 ± 0.088</td>
</tr>
<tr>
<td>Spring red wheat (F.S.)</td>
<td>1.007 ± 0.221</td>
</tr>
<tr>
<td>Row barley (F.S.)</td>
<td>0.975 ± 0.428</td>
</tr>
<tr>
<td>Ethanol (1%)</td>
<td>0.695 ± 0.076</td>
</tr>
<tr>
<td>Lentils (F.S.)</td>
<td>0.796 ± 0.248</td>
</tr>
<tr>
<td>Brown rice (F.S.)</td>
<td>0.741 ± 0.130</td>
</tr>
<tr>
<td>Wild rice (F.S.)</td>
<td>0.736 ± 0.060</td>
</tr>
<tr>
<td>Maize (F.S.)</td>
<td>0.814 ± 0.419</td>
</tr>
</tbody>
</table>

* Values are the means ± S.D. (n=3). Quantification was done by densitometry. None of the samples were statistically different than control. F.S.= full strength.
DISCUSSION

Phytochemicals present in fruits, vegetables and whole grains have the potential to inhibit the development of cancer. Among these phytochemicals, flavonoids and other polyphenolics present in fruits and vegetables have been the center of extensive study in the past. Several mechanisms have been proposed for their protective properties. Flavonoids and other polyphenolics can block the initiation of cancer by inhibiting phase I enzymes, inducing phase II enzymes, and by scavenging reactive oxygen species (ROS) that can react with DNA and cause mutations. They can also modulate the promotion and progression of cancer by altering apoptosis and angiogenesis (19, 98).

In the present work, we studied the potential cancer preventive properties of flavonoids and other polyphenolics present in various cereal grains. Cereal grains are a major component of western diets. In the past, cereal grains and legumes have been studied mainly for their fiber content and their beneficial effects in the protection against colon cancer (2). However, little attention was paid to the possible protective effect of the phenolic fraction of those grains. Cereal grains and legumes are a rich source of phenolics, providing approximately 44 mg of flavonoids per day in the diet (15). Some of these polyphenolics have shown antioxidant activity in vitro (22) and have the potential of being cancer preventive (reviewed in (113)). We were interested in studying the potential cancer preventive properties of the flavonoids present in a variety of cereal grains. Our goal was to study the capacity of the various flavonoid extracts to block the initiation of cancer by inducing NQO1, and to inhibit the promotion and progression of cancer by inhibiting COX-2.
ROS generated in the metabolism of carcinogens can cause DNA damage, which is an important factor in cancer initiation. Animals have developed antioxidant defense systems against toxic compounds. Induction of Phase II enzymes such as GST, NQO1 and UDP-GT, and elevated levels of glutathione are examples of these antioxidant defense mechanisms (25). NQO1 is a two-electron quinone reductase that reduces quinones directly to hydroquinones, avoiding the formation of free radicals generated in the redox cycling of an intermediate semiquinone (27, 29, 30). Induction of Phase II detoxification enzymes by natural or synthetic compounds is a well-established strategy towards cancer chemoprevention (25, 26, 33-35). The induction of NQO1 activity has protective effects against toxicity, mutagenesis and carcinogenesis (33, 35-37).

Animal and epidemiological studies have shown the potential of flavonoids as cancer chemopreventive agents. The cancer-protective effects of flavonoids have been associated, in large part, with their antioxidant properties. Besides their ability to scavenge free radicals, flavonoids can also act as chelators of metal ions and as modulators of antioxidant enzymes (114). The latter property raised our interest. In particular, we were interested in the capacity of flavonoids from different grains to stimulate the activity of NQO1.

Preliminary studies of NQO1 activity (Figure 8) showed a significant induction of the enzyme after a 48 h exposure to the first set of polyphenolic extracts. Hepalclc7 cells were exposed to 10-fold dilutions of the lentils, kamut, spring hard wheat, spring red wheat, winter wheat, dehulled barley, and spelt extracts. One hundred-fold dilutions of the extracts of oats, rye, row barley, maize, wild rice, and brown rice were used due to the toxicity observed with more concentrated solutions. In the wheat group, induction rates
ranged from a 1.6-fold induction with spring red wheat to a 3.9-fold induction with spelt. The induction observed with spelt polyphenols is quite remarkable when compared to the NQO1 induction observed with standard phenolic inducers, such as t-BHQ and HQ (28). Exposure of t-BHQ (20µM) and HQ (20 µM) to Hepa1c1c7 cells for 48 h resulted in a 4.2- and 3.2-fold induction in the enzyme’s activity, respectively (Table 3). Another interesting observation was the difference between the NQO1 induction rates after incubation with the extracts of row barley and dehulled barley. Row barley, the most unprocessed form of barley tested, produced a 2.6-fold induction of the enzyme’s activity, whereas dehulled barley produced a 1.4-fold induction. Also outstanding is the difference in enzyme induction observed between wild rice and brown rice. A 2.7-fold induction was obtained with wild rice, but only a 1.3-fold induction was observed with brown rice. In most refining processes, phytochemicals and nutrients present in the outer parts of the grains are removed, reducing the nutritional value of the grains (reviewed in (10)). The NQO1 results obtained in these experiments suggest that their potential cancer preventive properties may also be reduced during the refining process. A 2.5, 2.4, 2.9, and 1.4-fold induction of NQO1 activity was observed with the extracts of lentils, oats, rye and maize, respectively. The NQO1 induction observed with maize and brown rice was not significant.

When experiments were repeated with a new set of extracts, a significant NQO1 induction (Figure 9) was observed in Hepa1c1c7 cells after a 48-h exposure to a 10-fold dilution of the wild rice, lentils, spring red wheat and winter wheat extracts. A 1.5, 1.6, 1.5, and 1.8-fold NQO1 induction was observed with the flavonoids of spring red wheat, winter wheat, wild rice, and lentils, respectively. In the second set of extracts, the residue
obtained after evaporation of the ethyl acetate fraction was dissolved in 5 ml of 100% ethanol and filtered to eliminate the insoluble residue. Results from the phenolic quantification of the extracts (Figures 5, 6, and 7) show that dehulled barley and row barley have the highest concentrations in total phenolics, with concentrations of 10.3 mM and 9.8 mM, respectively. All types of wheat ranged from 0.3 mM to 0.6 mM, containing the lowest polyphenolic concentrations of the grains analyzed. Wild rice, the most unprocessed form, contains a phenolic concentration 5 times higher than brown rice (3.7 mM vs 0.7 mM). Oats, rye, lentils and maize have a phenolic content of 1.2 mM, 0.9 mM, 3.9 mM, and 1.5 mM, respectively. No correlation between the amount of polyphenolics and NQO1 induction was observed.

One possible explanation for the results observed in these experiments is that the concentration of flavonoids in some extracts of the second set may be insufficient to cause a significant NQO1 induction. In the literature, a wide range of concentrations of flavonoids and other polyphenolics caused induction of NQO1. Exposure of quercetin (15μM) to a human breast carcinoma cell line (MCF-7) for a period of 24 h, resulted in a two-fold increase in NQO1 activity (46). The polyphenolic resveratrol doubled NQO1 activity in Hepalc1c7 cells at a concentration of 21 μM (28). Enterolactone and genistein doubled the activity of NQO1 in colonic Colo 205 cells at concentrations of 0.04 and 0.14 μM, respectively. A 2 to 3-fold NQO1 induction was observed in the same cell line with biochanin A (1.1 μM) and coumestrol (12.0 μM) (42). The flavanone 6,8-diprenylnaringenin present in hops doubled NQO1 activity at a 4.4 μM concentration in Hepalc1c7 cells (115).
Although lower concentrations of flavonoids have been previously shown to induce NQO1, the flavonoids contained in our extracts may not be potent enough to stimulate NQO1 at these concentrations. Flavonoid glycosides lost in the extraction process may also contribute to NQO1 induction. Collection of the aqueous and lipid fractions should be considered in future studies. There is also the possibility of losing active compounds during the filtration of the extracts. This last hypothesis would explain the differences seen in the enzyme’s activity with the two sets of extracts.

In future studies, serial dilutions of the extracts should be made to assure that the lack of NQO1 induction is not due to a low flavonoid concentration. An assay for detection of NQO1 inducers described by Prochaska et al. (28), may be useful for this purpose. In that assay, Hepa1c1c7 cells are plated in 96 well plates, and incubated for 24h with the potential anticarcinogenic agents. Cells are lysed and assayed in a solution containing 2-methyl-1,4-naphthoquinone (menadione), [3-(1,5,-di-methylthiazo-2-yl)-2,5-diphenyltetrazolium bromide] (MTT), and a NADPH-generating system. NQO1 reduces menadione to menadiol, using NADPH as a cofactor. Menadiol spontaneously reduces MTT to formazan dye. The production of the formazan dye can be monitored spectrophotometrically at 610 nm, and used as a measure of NQO1 activity (28).

We were also interested in another approach towards cancer chemoprevention with dietary flavonoids: the inhibition of COX-2. COX-2, a rate-limiting enzyme in the biosynthesis of prostaglandins from AA, has also raised interest as a possible way of inhibiting cancer growth. Observations from epidemiological studies have shown that aspirin and other NSAIDs reduced the risk of colorectal cancer by 40-50% (reviewed in (61)). Sulindac, a COX inhibitor, also reduced the size and number of polyps in patients
with FAP (reviewed in (61)). More evidence of the involvement of COX-2 in
tumorigenesis came from the studies of Oshima et al. (82), in a mouse model for human
FAP. A reduction in the number and size of intestinal polyps was observed when the gene
encoding for COX-2 was disrupted, and when mice were treated with a COX-2 selective
inhibitor (82). COX-2 is upregulated in many tumors, where it may play a critical role in
the formation of blood vessels (116). The formation of new blood vessels is essential for
the delivery of oxygen and nutrients to the tumor and ultimately for tumor growth. The
degree of vascularization of a tumor is related to its invasiveness and metastatic potential
(reviewed in (85)). PGE$_2$ has been shown to induce the formation of new blood vessels in
the chorioallantoic membrane model (94). PGE2 stimulated the expression of VEGF in
synovial fibroblasts (95) and in pre-osteoblastic cells (96). VEGF and bFGF transcription
were also stimulated by PGE$_2$ in cultured rat Müller cells (97).

Flavonoids have previously been shown to possess antiproliferative and
antiangiogenic properties in vitro by blocking bFGF- and VEGF-induced angiogenesis
(98). Polyphenols from green and black tea, and theaflavins inhibited COX-dependent
arachidonic acid metabolism in normal colon cells by 37-62 % at 30 µg/ml (101).
Possible chemopreventive compounds, such as quercetin, genistein, kaempferol,
resveratrol and resorcinol, sharing a resorcin-type structure, suppressed COX-2
transcription in colon cancer cells (102).

Preliminary studies with the first set of extracts showed COX-2 inhibition with
oats and rye polyphenols (Figure 10), suggesting the potential of these compounds to
inhibit carcinogenesis at the promotion and progression step. Further research with the
second set of extracts was conducted to investigate this effect. Two different extract
dilutions were used to evaluate the inhibition of COX-2 expression in U87 cells. In both cases, cells were exposed to the polyphenolic extracts for a period of 48 hours. A final concentration of 2 μM-100 μM of the polyphenols was obtained when using the extracts at full strength, and a concentration of 0.2 μM -10 μM when a 10-fold dilution was prepared. As shown in Figures 11 and 12, there was no inhibition of COX-2 expression observed when using a 10-fold dilution of the extracts. Extracts at full strength were used as an attempt to observe the inhibition of COX-2 expression, and to assure that the lack of inhibition was not due to a low concentration of the polyphenols in the extracts. When using the polyphenolic extracts at full strength (Figure 13 and 14), there was also no COX-2 inhibition observed. The quantification of the bands by densitometry (Tables 6, 7, 8, and 9) confirmed the results observed in the Western blots.

One possible explanation for these results is that potentially active compounds present in the extracts may be lost during the filtration process. With the extraction method utilized, flavonoid glycosides were also eliminated in the aqueous fraction. In the literature, flavonoid aglycones appear as the main inhibitors of COX-2 enzymatic activity in vitro, whereas flavonoid glycosides are less inhibitory or non-inhibitory (117, 118). Although flavonoid glycosides do not inhibit COX-2 activity in vitro, they may play an important role in vivo, as they are better absorbed from the diet than the flavonoids aglycones (48). A study by Reddy et al. (100), also showed that the oil fraction of wheat bran had cancer protective effects. Wheat bran oil inhibited the activity and expression of COX-2 and inducible nitric oxide synthase (iNOS) in colon tumors. The incidence, size and multiplicity of AOM-induced colon tumors in rats were also reduced when compared to animals fed with the non-fortified standard diet (100). Lipid soluble components of the
cereal grains tested may be involved in COX-2 expression inhibition. With the extraction method employed, the lipid soluble compounds are removed in the chloroform extraction. Collection of an aqueous fraction, a lipid soluble fraction and an ethyl acetate fraction of the extracts should be considered in future studies. Another observation made in the culture of the U87 cells was that the flavonoid extracts of oats and dehulled barley were toxic when they were used at full strength. The cytotoxic properties of flavonoids and other polyphenols have been observed (115, 119, 120), and some flavonoids, such as chalcones and flavanones from hops, have been studied as potential anti-neoplastic agents (115, 120).

We initiated this project with the goal of investigating the potential cancer preventive properties of the various grain extracts. We opted for a general study of the nonpurified flavonoid fraction of the grains, instead of studying the properties of a single flavonoid present in them. In many previous studies the latter approach was taken to study the cancer chemopreventive mechanisms of flavonoids. Genistein from soy, resveratrol from grapes and quercetin are examples of polyphenols studied extensively in this way. We were interested in a method that would allow us to determine the chemopreventive potential of the grain extracts as a whole. This approach would also let us evaluate the need for further research in the single components of these extracts. One disadvantage of our method is that other compounds present in the extracts may also contribute to the effect observed. For example, the lack of COX-2 inhibition observed may be due to an opposite inducing effect of one or more components of the extracts. Isolation of different sub-fractions should be considered in future to evaluate this possibility.
In conclusion, this project studied the potential cancer preventive properties of flavonoids from cereal grains and legumes. We explored the inhibition of the carcinogenic process at the initiation step, by inducing a Phase II detoxification enzyme, NQO1, and at the promotion and progression step, by inhibiting COX-2. The results obtained in the NQO1 studies suggest that the extracts from cereal grains and legumes have the potential to be cancer chemopreventive. Preliminary studies with the first set of extracts showed NQO1 induction with most of the extracts. In experiments conducted with the second set of extracts, only spring red wheat, winter wheat, wild rice and lentils induced NQO1 activity. The reduction in significance seen with the new extracts suggests that at some point in the new extraction method we removed an important fraction of the extracts. Identification of compounds in such fractions should be considered in future studies. Further research is needed to evaluate the polyphenolic concentration necessary to cause a significant NQO1 induction. For that purpose, we will prepare new extracts starting with a larger quantity of each grain and prepare serial dilutions of the extracts.

Results from COX-2 immunoblotting failed to show a significant COX-2 inhibition when cells were exposed to a 1x and 10x dilution of the second set of extracts. Preliminary work with the first set of extracts showed COX-2 inhibition with the extracts of oats and rye, suggesting that compounds present in the first set of extracts may be lost in the filtration of the second set of extracts. Collection of the aqueous and lipid fractions of the extracts should also be considered in the future.

In future work, we will focus on the fractionation of the grain extracts and the isolation of the different components. We will also study the chemopreventive effects of the grain extracts in vivo, using an animal model susceptible to tumors. The number and
size of the tumors in the animals fed with diets fortified with the various extracts will be used as a measure of cancer protection. This would allow us to study the efficacy of grain extract fractions in protecting against cancer when administered through the diet.
BIBLIOGRAPHY


39. Wefers, H., Komai, T., Talalay, P., and Sies, H. Protection against reactive oxygen species by NAD(P)H: quinone reductase induced by the dietary


44. Kelly, V. P., Ellis, E. M., Manson, M. M., Chanas, S. A., Moffat, G. J., McLeod, R., Judah, D. J., Neal, G. E., and Hayes, J. D. Chemoprevention of aflatoxin B1 hepatocarcinogenesis by coumarin, a natural benzopyrrole that is a potent inducer of aflatoxin B1-aldehyde reductase, the glutathione S-transferase A5 and P1 subunits, and NAD(P)H:quinone oxidoreductase in rat liver, Cancer Res. 60: 957-69., 2000.


101. Hong, J. I., Smith, T.J., Ho, C.T., August, D.A., Yang, C.S. Effect of green and black tea polyphenols on cyclooxygenase (COX) and lipooxygenase (LOX)-dependent metabolism of arachidonic acid in human colon tissues, The toxicologist. 59; 2000.


