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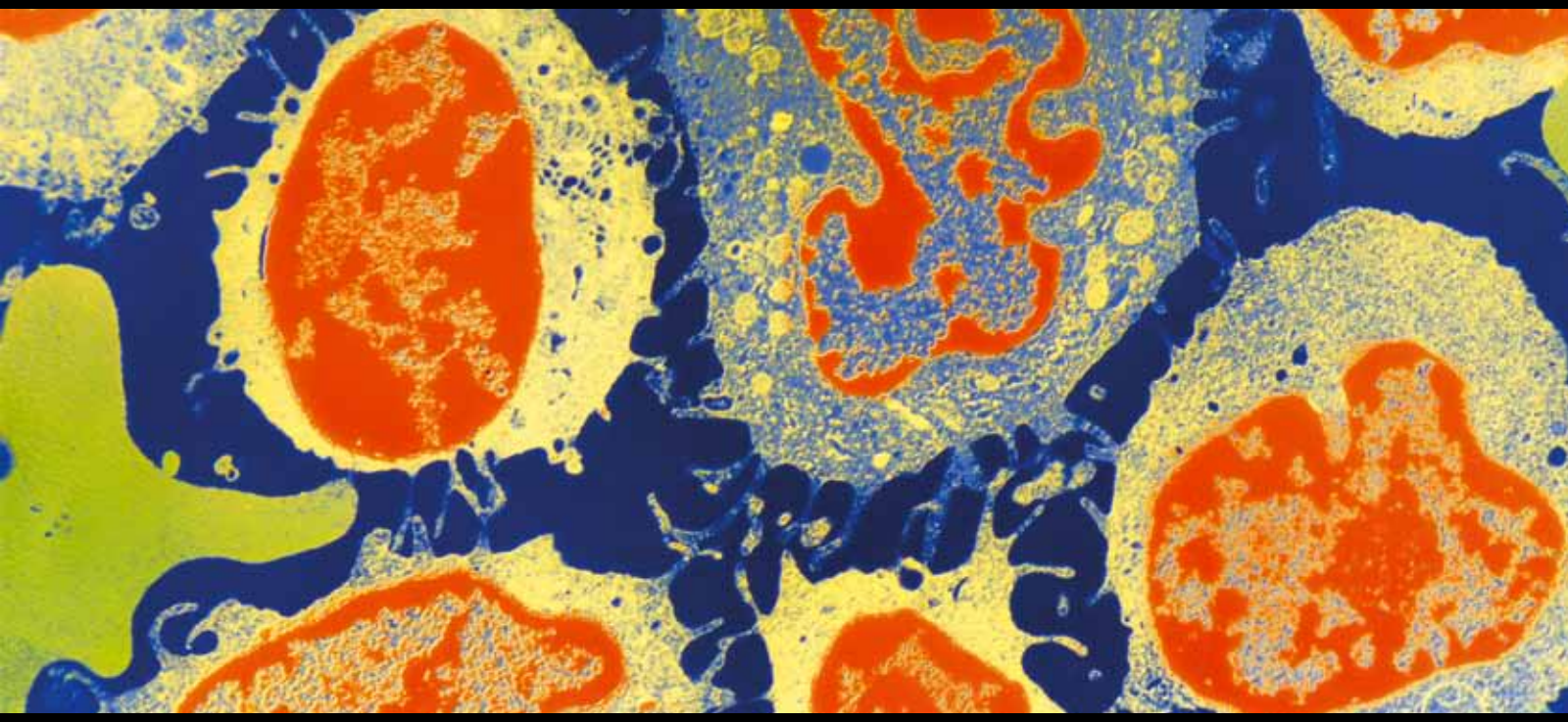
Dietary Agents in Cancer Chemoprevention and Treatment

Guest Editors: Julian J. Raffoul, Omer Kucuk, Fazlul H. Sarkar, and Gilda G. Hillman



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Editorial Board

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Editorial

Dietary Agents in Cancer Chemoprevention and Treatment

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Cancer chemoprevention using natural or synthetic compounds to prevent or suppress the development of cancer, is an area of active investigation. Many compounds belonging to diverse chemical classes have been identified as potential chemopreventive agents, including vitamins and minerals, naturally occurring phytochemicals, and synthetic compounds. Understanding the molecular mechanisms of cancer chemoprevention is not only important for the safe application of these compounds in populations of patients at high risk for cancer, but also allows for further development of novel treatment regimens for cancer patients.

This special issue contains original research as well as review articles that are intended to stimulate the continuing efforts to understand the use of dietary agents in cancer chemoprevention and treatment. The lead article by S. N. Saldanha and T. O. Tollefsbol provides a comprehensive review of dietary agents that have shown strong chemopreventive and therapeutic properties *in vitro*. They also discuss the design and modification of these bioactive compounds for pre-clinical and clinical applications.

Dietary intake of foods rich in antioxidant compounds has been suggested to be cancer protective. However, randomized clinical trials and epidemiologic studies on the association between intake of foods rich in antioxidants and cancer incidence have yielded mixed results. M. Y. Wei and E. L. Giovannuci discuss the epidemiologic considerations of lycopene as a chemopreventive agent, including measurement of lycopene, its major source in the diet, and the assessment of prostate cancer incidence and progression, with particular emphasis on the effect of PSA screening on this association. K. Zhou and J. J. Raffoul discuss the composition and cancer-protective effects of major phenolic antioxidants in grape skin and grape seed extracts. M. A. Parasramka and S. V. Gupta provide original research demonstrating the anticancer properties of garcinol alone, or combined with curcumin, on pancreatic cancer cells. Garcinol, a polyisoprentylated benzophenone extracted from the rind of the fruit *Garcinia indica*, a plant found in tropical regions, has antioxidant and anti-inflammatory properties and its role as anticancer agent is thoroughly discussed in the review from N. Saadat and S. V. Gupta.

Two manuscripts discussing the effect of dietary agents on DNA repair capacity are also part of this special issue. In a manuscript by J. J. Raffoul et al., the potential for targeting the DNA base excision repair enzyme APE1/Ref-1 using dietary agents such as soy isoflavones, resveratrol, curcumin, ascorbate, and alpha-tocopherol is discussed. The potential for these natural compounds to be combined with chemotherapy or radiotherapy for the more effective treatment of cancer are also reviewed. A proposed mechanism of action is discussed and an attempt is made to delineate which of the two activities of APE1/Ref-1 (DNA repair versus redox activation of cellular transcription factors) is responsible for the observed effects. The second manuscript by R. Rosati et al. reviews the role for dietary folate in the prevention of colorectal cancer. Data are presented which demonstrate that inhibition of DNA repair is protective in the development of preneoplastic colon lesions, both when folate is depleted and when it is not. This manuscript is a comprehensive review of the literature and provides a critical analysis of

the experimental designs used in folate and colorectal cancer research.

Two additional manuscripts detailing the ability of dietary agents to sensitize cancer cells to chemotherapy and radiotherapy are included in this special issue. An original research article by S. Duangmano et al. demonstrates that curcurbitacin B, a plant phytochemical, inhibited breast cancer cell proliferation in a dose-dependent manner and caused radiosensitization of human breast cancer cells via G2/M cell cycle arrest. Furthermore, an original research article by K. Sahin et al. demonstrate that genistein, a soy isoflavone, sensitizes cervical cancer cells to cisplatin via inhibition of NF-kappa B and Akt/mTOR cell signaling pathways.

This special issue concludes with a report of a clinical study demonstrating the prevention of anthracycline-induced cardiac toxicity through supplementation with selenium in a group of pediatric cancer patients.

Research efforts aimed at understanding the role of dietary agents and phytochemicals in cancer prevention and treatment are likely to yield high-impact results that have the potential for immediate clinical applications. Furthermore, combination of phytochemicals and nutritional agents with therapies for advanced cancers, including radiotherapy and chemotherapy, would benefit from a complementary and safe approach using dietary agents to mitigate the adverse effects of these therapies on normal tissues while enhancing the therapeutic efficacy. Elucidation of the mechanisms of interaction between dietary agents and conventional cancer treatments will have a major impact on understanding the molecular mechanisms of cancer chemoprevention and will ultimately result in clinical use of dietary agents as an adjunct to standard cancer treatment.

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Clinical Study

Selenium in the Prevention of Anthracycline-Induced Cardiac Toxicity in Children with Cancer

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High cumulative doses of anthracyclines (300–500 mg/m^2) used in the treatment of children with cancer may result in cardiotoxicity, a major long-term adverse effect that limits clinical usefulness of this class of chemotherapeutic agents. We assessed anthracycline-induced cardiotoxicity by measuring Pro-BNP levels and echocardiographic (ECHO) findings and investigated potential protective effect of selenium (Se) supplementation in a group of pediatric cancer patients. Plasma level of Pro-BNP was measured, and ECHO was performed in 67 patients (45 boys, 22 girls; ages 2–18 years; median age 12 years) after they completed anthracycline-containing chemotherapy. Serum Se level was measured in 37 patients. Eleven patients had high Pro-BNP levels and/or cardiac failure with Pro-BNP levels of 10–8,022 pg/mL (median 226.3 pg/mL; laboratory normal level is less than 120 pg/mL). Serum Se levels were low (20–129 mcg/L, median 62 mcg/L) in ten of these eleven patients. Eight of 10 patients with low Se and high Pro-BNP levels were supplemented with Se 100 mcg/day for a period of 4–33 months (median 6 months) which resulted in improvement in Pro-BNP and/or ECHO findings. These results suggest that Se supplementation may have a role in protection against anthracycline-induced cardiac toxicity.

1. Introduction

High cumulative doses of anthracyclines (300–500 mg/m^2) are frequently administered to children with cancer. Cardiac toxicity is a serious adverse effect that limits the therapeutic potential of anthracyclines and threatens the cardiac function of pediatric cancer patients leading to debilitating long-term effects resulting in poor quality of life in cancer survivors [1–5]. This is particularly devastating in children who are cured of their cancer because they have to endure the debilitating cardiac dysfunction for the rest of their lives with limited exercise capacity which may also lead to other chronic illnesses.

B-type-natriuretic peptide (BNP) is a polypeptide hormone predominantly released from the cardiac ventricles in response to volume expansion and pressure overload. BNP is found in the circulation as BNP-32 and the NH_2-terminal portion of ProBNP (Nt-proBNP). BNP levels are elevated in patients with left ventricular systolic dysfunction and correlate with the severity of symptoms and prognosis [6–14]. Measuring serum Pro-BNP levels is a reliable way to monitor the cardiac function of patients receiving cardiotoxic drugs such as anthracyclines.

Selenium (Se) is a trace element distributed in a small amount in the soil and certain foods. It is an important

antioxidant, and its absence has been associated with cardiomyopathy in people living in areas with poor levels of soil Se. The concentration of Se in grain varies based on the soil content. Dietary Se is found in meat and seafood. It is a cofactor for glutathione peroxidase which catalyzes the reduction of hydrogen peroxide using glutathione. It is an essential element to remove free radicals from the body and to prevent oxidative tissue damage [15–19]. Se supplementation could potentially prevent cardiac toxicity of anthracyclines [16–20].

In this study, we assessed anthracycline-induced cardiotoxicity by measuring Pro-BNP levels and echocardiographic (ECHO) findings, and we investigated the potential protective effect of Se supplementation in a group of children with high Pro-BNP levels and/or cardiac dysfunction.

2. Patients and Methods

Plasma level of Pro-BNP was measured, and echocardiography (ECHO) was performed in 67 pediatric cancer patients (45 boys and 22 girls, ages between 2 and 18 years, median age 12 years) with a variety of tumors (leukemias, lymphomas, solid tumors) after completing anthracycline-containing treatment. Serum Se levels were measured in 37 patients. Sera were stored at −20 degrees centigrade until selenium levels were measured with atomic absorption method. Patients with low level of Se were supplemented with Se (100 mcg/day).

3. Statistical Analysis

Statistical analysis was performed using SPSS (Version 15.0) software package. Comparisons between the groups were done using Mann-Whitney U test, Wilcoxon sign test, and Fisher's exact test. Levels of statistical significance were set at a P value < 0.05. The results were expressed as range (minimum and maximum) and median.

4. Results

In eleven patients who had high Pro-BNP levels and/or cardiac failure Pro-BNP levels ranged between 10 and 8022 pg/mL with a median of 226.3 pg/mL (normal < 120 pg/mL). Fifty-six patients had normal Pro-BNP levels (8.2–119.6 pg/mL, median 32.4 pg/mL). As seen in Table 1, the difference in levels of Pro-BNP between these two groups was significant ($P < 0.001$). Serum Se levels were low in 10 of these 11 patients with high Pro-BNP levels and/or cardiac failure (20–129 mcg/L, median 62 mcg/L). Twenty-six of 56 patients with normal Pro-BNP levels were also investigated for Se levels (51.3–150 mcg/L, median 99.4 mcg/L). There was a significant difference between Se levels of patients in high Pro-BNP and normal Pro-BNP groups ($P < 0.001$) (Table 1).

Abnormal ECHO findings were observed in 7 of 11 (63.6%) patients with high Pro-BNP levels and/or cardiac failure group. Only 1 (3.8%) of 26 patients with normal Pro-BNP levels had abnormal ECHO finding. A patient with normal pro-BNP and low Se level died in 1 month because of progressive disease with respiratory failure and cardiac failure. The probability of having abnormal ECHO findings was significantly higher in patients with high Pro-BNP compared to those with normal Pro-BNP ($P < 0.001$) (Table 2). Eight of 11 patients with low Se and high Pro-BNP levels were supplemented with Se 100 mcg per day for 4–33 months (median 6 months). Three of 8 patients had cardiac failure according to ECHO and were supplemented with Se in addition to digoxin and ACE inhibitors. All 3 patients were doing well with normal ECHO findings and normal Pro-BNP levels after a follow-up periods of 33, 14, and 5 months. Five patients, 3 with normal ECHO and 2 with diastolic dysfunction (one with low Pro-BNP level, other with high Pro-BNP level) also, were supplemented with selenium (100 mcg per day). One patient who had diastolic dysfunction with normal Pro-BNP did well with Se supplementation with normalization of ECHO findings, but she later died due to progression of her cancer. Another patient with diastolic dysfunction as well as 3 patients with normal ECHO had normal Se and Pro-BNP levels after 4–6 months of Se supplementation. Only 3 patients were not supplemented with Se in the high Pro-BNP and/or cardiac failure group, because one of them had normal Se level, the second one died with progressive disease in a very short period of time, and the third one had Pro-BNP level within normal limits after the removal of intracardiac tumor thrombus with open heart surgery (Table 3). In Se-supplemented group, supplementation period was between 4 and 33 months (median 6 months). Before supplementation, Pro-BNP levels were between 10 and 843 pg/mL (median 175 pg/mL). After supplementation, Pro-BNP levels were 2–536 pg/mL (median 73.5 pg/mL) which were significantly lower than pretreatment levels ($P = 0.018$). Pretreatment Se levels were between 20 and 83 mcg/L (median 57 mcg/L). After supplementation Se levels were 65–109 mcg/L (median 103 mcg/L) which were significantly higher than presupplementation level ($P = 0.028$) (Table 4). After achieving normal Se and Pro-BNP levels, Se supplementation was discontinued. During follow-up period with no Se supplementation, 2–6 months after supplementation repeat measurements of Se levels were 75–106 mcg/L (median 83 mcg/L), and Pro-BNP levels were 10–123.5 pg/mL (median 106.5 pg/mL), which were lower for Se ($P = 0.068$) and higher for Pro-BNP ($P = 0.109$) compared to Se-supplemented period (Table 4).

Table 1: Selenium and Pro-BNP levels of patients.

	Normal Pro-BNP ($n = 56$)		High Pro-BNP and/or abnormal ECHO ($n = 11$)	
	Range	Median	Range	Median
*Pro-BNP (pg/mL)	8.2–119.6	32.4	10–8022	226.3
Selenium (mcg/L)*	51.3–150	99.4	20–129	62

* $P < 0.001$.
** $P < 0.001$.
*** $n = 26$ (twenty-six of fifty-six patients with normal Pro-BNP were measured for Se level) Mann Whitney U test was used, and median (range) was given as descriptive statistics.

Table 2: Echo findings of patients with high and normal Pro-BNP levels.

	ECHO findings		Total number of patients (%)
	Normal $n = 29$ (%)	Abnormal $n = 8$ (%)	
Normal Pro-BNP levels	25 (96.2)	1 (3.8)	26 (100)
High Pro-BNP levels	4 (36.4)	7 (63.6)	11 (100)
Total	29 (78.4)	8 (21.6)	37 (100)

$P < 0.001$ (Fisher's exact test).

5. Discussion

The main long-term toxicity of anthracyclines is cardiac dysfunction associated with their chronic and/or high-dose administration. Severe cardiomyopathy and congestive heart failure may develop any time after the completion of the treatment. The precise pathogenesis of anthracycline-induced cardiotoxicity is still uncertain, and it is likely to be multifactorial in origin. Nevertheless, pivotal role is attributed to the iron-catalyzed intramyocardial production of reactive oxygen species (ROS), which cause damage of various targets in the myocardial cells [1–5]. Probrain natriuretic peptide (Pro-BNP) is released by cardiac cells, and serum levels are elevated even before the development of overt cardiac distress symptoms related to impairment of left ventricular systolic or diastolic function leading to increased left ventricular wall stretch. Recent studies have also suggested that ischemia itself may promote release of BNP [7, 20–24]. In the present study, we evaluated cardiotoxicity in 67 pediatric patients with cancer (leukemia, lymphoma, and solid tumor) after they completed treatment with anthracycline-containing regimens. We also evaluated Se levels and the effects of Se supplementation with regard to cardiotoxicity because previous studies with Keshan disease (KD) suggested potential protective role of Se for cardiac dysfunction observed in Se deficiency.

KD, a potentially fatal form of cardiomyopathy, first found in Keshan county, northeast China, is one of the most harmful endemic diseases. The disease is characterized by multifocal myocardial necrosis and fibrosis and leads to congestive heart failure and cardiogenic shock. Although the exact etiology of KD is unclear, Se deficiency is a major contributing factor [19]. Investigations into the epidemiology of KD revealed that individuals living in areas with Se-poor soil were under a high risk of development of the disease. Individuals living in those areas had low dietary intakes of Se that were reflected in low serum and hair levels of Se. Populations living in areas of China with Se-rich soil did not develop KD [25–28].

In this study we have investigated the potential role of Se in anthracycline-induced cardiotoxicity in pediatric cancer patients undergoing chemotherapy. We found an association between low Se levels and anthracycline cardiotoxicity which could be prevented by Se supplementation. These results suggest that Se deficiency may have an effect on anthracycline-induced cardiomyopathy, which may have similarities to KD.

The family of selenoproteins includes glutathione peroxidases, the redox enzymes that take advantage of the chemical properties of Se to remove free radicals by reduced glutathione and thus to form oxidized glutathione. Se supplementation had a protective effect on ischemia/reperfusion injury in experimental animals; it improved the recovery of cardiac function, decreased ultrastructural changes, increased the expression of glutathione-related enzymes, and partially affected the antioxidant capacity of the tissues together with an effect on gene transcription level [29, 30]. Se supplementation prevented the hypoxia/reoxygenation injury of the isolated neonatal cardiomyocytes and resulted in an NO-related increase of inotropic response of cardiac muscle to the beta-adrenergic stimulation by isoproterenol [17]. Oral Se supplementation has been shown to reverse the biochemical evidence of the Se deficiency [29–31]. The beneficial effect of treatment with the inorganic form of Se was also demonstrated in experimental models of cardiac injury [31, 32]. The mechanism by which Se influences iNOS cardiac expression is unknown. Kim et al. [33] have shown that lipopolysaccharide-activated human T cells with relatively high concentrations of selenite had lower NF-kB-binding and -decreased NO production. Similarly, Turan et al. [34] observed that total NF-kB in the cardiac muscle was reduced by Se. They suggested that Se deficiency or excess affects signal transduction. Se effect can be monitored with Pro-BNP, a good marker of cardiac function [7, 35].

Dietary supplementation of 100 μg Se (sodium selenite) in patients receiving total parenteral nutrition has been reported to prevent arrhythmias and cardiomegaly and lead to an increase in left ventricle ejection fraction [36]. In addition, the incidence of Keshan disease, an endemic dilated congestive myocardiopathy in areas of Se deficiency in China and Russia, has been shown to be decreased by oral Se supplementation at a dosage of 150–300 μg/week [36, 37]. It should be noted that Se supplementation has also been suggested as a strategy for prevention of myocardial disease in other studies of human cardiac pathology [36–38].

The results of our study support the hypothesis that Se supplementation could be considered as a strategy for treatment and prevention of anthracycline-induced cardiomyopathy observed in children with cancer. Our results also suggest that Se supplementation should be continued much longer to ameliorate or prevent anthracycline-induced cardiotoxicity. In conclusion, our results suggest that Se supplementation may have a potential role in the protection against anthracycline-induced cardiac toxicity in patients with high pro-BNP level and/or cardiac failure and low Se levels.

Table 3: Se-supplemented patients with low serum Se levels, high Pro-BNP levels, and/or cardiac failure.

Pt. $n = 11$	Total anthracy mg/m^2	Pro-BNP pg/mL	Se mcg/L	ECHO	Cardiac failure	Digoxin Enalapril Furosem	Se Suppl.	Echo	End results (i) Pro-BNP (pg/mL)	End results (ii) Pro-BNP (pg/mL)	End results (i) Se (mcg/L)	End results (ii) Se (mcg/L)
ES	550	754	70	Systolic failure	+	+	100 mcg	Normal	536	123.5	108	86
NBL	180	175	52	Systolic failure	+	+	100 mcg	Normal	85	95	103	106
ACC	400	10	71	Diastolic failure	–	–	100 mcg	Normal	10	10	65	75
HL	300	843	55	Diastolic failure	–	–	100 mcg	Normal	298	118	72	81
NHL	240	172	49.2	Normal	–	–	100 mcg	Normal	12.6	NA	109	NA
HL	400	197.5	20	Normal	–	–	100 mcg	Normal	80	NA	208	NA
BL	150	170.4	57	Normal	–	–	100 mcg	Normal	2	NA	75	NA
NHL	120	277	83	Systolic failure	+	+	100 mcg	Normal	NA	NA	NA	NA
RMS	0	127	62	Intracardiac thrombus	–	–	—	Normal	67	NA	NA	NA
AML	400	8022	65	Normal	–	–	—	Died	NA	NA	NA	NA
TALL	320	1536	129	Pericardial effusion, tamponade	–	–	—	Died	NA	NA	NA	NA

Pt: patient; NA: not available, ES: Ewing's sarcoma, NBL: neuroblastoma, ACC: adrenocortical carcinoma, HL: Hodgkin lymphoma, NHL: non-Hodgkin lymphoma, BL: Burkitt lymphoma, RMS: rhabdomyosarcoma, AML: acute myeloid leukemia; T ALL: T acute lymphoblastic leukemia.

Table 4: Pre- and postsupplementation levels in Se-supplemented patients with low serum Se levels and high Pro-BNP levels and/or cardiac failure.

$n = 8$	Presupplementation levels, range (median)	1st postsupplementation levels, range (median)	2nd postsupplementation levels, range (median)
Pro-BNP (pg/mL)	10–843 (175)	2–536 (73.5)*	10–123.5 (106.5)**
Se (mcg/L)	20–83 (57)	65–109 (103)***	75–106 (83)****

*$P = 0.018$.
**$P = 0.109$.
***$P = 0.028$.
****$P = 0.068$.
Wilcoxon sign test was used; median and range were given as descriptive statistics.

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Review Article

Folate and Colorectal Cancer in Rodents: A Model of DNA Repair Deficiency

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Fortification of grains has resulted in a positive public health outcome vis-a-vis reduced incidence of neural tube defects. Whether folate has a correspondingly beneficial effect on other disease outcomes is less clear. A role for dietary folate in the prevention of colorectal cancer has been established through epidemiological data. Experimental data aiming to further elucidate this relationship has been somewhat equivocal. Studies report that folate depletion increases DNA damage, mutagenesis, and chromosomal instability, all suggesting inhibited DNA repair. While these data connecting folate depletion and inhibition of DNA repair are convincing, we also present data demonstrating that genetic inhibition of DNA repair is protective in the development of preneoplastic colon lesions, both when folate is depleted and when it is not. The purpose of this paper is to (1) give an overview of the data demonstrating a DNA repair defect in response to folate depletion, and (2) critically compare and contrast the experimental designs utilized in folate/colorectal cancer research and the corresponding impact on tissue folate status and critical colorectal cancer endpoints. Our analysis suggests that there is still an important need for a comprehensive evaluation of the impact of differential dietary prescriptions on blood and tissue folate status.

1. Introduction

Folate deficiency has been linked to a variety of pathologic conditions and cancers. Perhaps most notably, folate is required during pregnancy for normal development of the neural tube closure. Once the connection between reduced dietary folate consumption and neural tube defects (NTDs) was well established, the FDA mandated fortification of grain-based foods with folic acid. This mandate resulted in a >25% decrease in incidence of NTDs in the United States [1]. This fortification resulted in a slight bump in average serum folate levels in the United States from approximately 12 ng/mL to approximately 19 ng/mL [2]. Normal range for serum folate concentration in humans is 2.7–17 ng/mL [3]. Thus, folate fortification has resulted in a positive public health outcome for its intended population, women of childbearing age, through moderate increases in serum folate levels and significant reduction in NTD incidence. However, folate is also strongly connected through epidemiological data to an increased risk to develop colorectal cancer. Unlike prevention of NTDs which targets young, healthy populations, colorectal cancer is primarily a disease of aging. Concern for whether folate fortification may be detrimental in this population group is born from rodent studies demonstrating a potentially negative effect of folate supplementation on disease pathology. The purpose of this paper is to evaluate this concern with a particular focus on the impact of folate depletion and supplementation in rodent models. We have focused on data evaluating colorectal cancer phenotypes.

Folate is ingested from food, primarily from fruits and vegetables in the form of polyglutamated folate, and from folate supplements (primarily folic acid), and is ultimately metabolised into a variety of oxidized and reduced forms with varying levels of methylation, thoroughly reviewed elsewhere [4]. The different folate forms are essential for purine synthesis, methionine remethylation (and therefore S-adenosyl methionine metabolism), and thymidylate synthesis, all of which can play important roles in genomic stability. The work from Fenech's lab has been instrumental

in establishing that chromosomal instability arises when folate is depleted, primarily in the form of micronuclei [5, 6]. Recently, Crasta et al. have demonstrated that micronuclei can induce further genomic instability through errors in chromosome segregation and genomic integration, as well as through chromothripsis, a process linked to carcinogenesis through massive chromosomal breakage and rearrangement [7]. These data provide a potentially direct mechanism for the carcinogenicity of folate depletion.

Micronuclei originate from acentric chromosomes, chromatid fragments, or whole chromosomes that fail to attach properly to the mitotic spindle during anaphase and therefore do not segregate properly during cytokinesis [5]. Experimental data demonstrates that folate depletion causes micronuclei formation [8–10], and genetic data likewise establishes a role for folate metabolism in micronuclei formation. SNPs in the reduced folate carrier (RFC) gene (G80A), the methionine reductase (MTR) gene (A2756G), and the MTHFR gene (C677T and A1298C) are associated with the formation of micronuclei [11–13]. Interestingly, SNPs in several DNA base excision repair genes have also been associated with micronuclei formation: OGG1 (C1245G), TDG (G595A), and XRCC1 (C26304T, G26466A, and G28152A) [14]. We suggest that a reduced ability to fully repair uracil in DNA results in an accumulation of DNA damage that promotes strand breakage and micronuclei formation.

2. Evidence That Folate Depletion Inhibits DNA Repair

Evidence collected from a variety of laboratories over the past decades has demonstrated an accumulation of DNA damage and/or mutations when folate is deficient. The mutagenic response to ENU (ethyl nitrosourea) is greater when folate is deficient [15]; EMS (ethyl methanesulfonate) and folate depletion induce a synergistic accumulation of DNA damage in Chinese hamster ovary (CHO) cells [16], and increased damage in response to MMS (methyl methane sulfonate) and hydrogen peroxide is seen in human colon epithelial cells when folate is depleted [17]; folate depletion makes human lymphocytes more sensitive to hydrogen peroxide [18], and more oxidative damage accumulates in response to amyloid β-peptide in neuronal cells depleted of folate [19]. These examples of accumulating damage point to an inability to repair the types of DNA damage repaired by the DNA base excision repair (BER) pathway. We directly tested the BER capacity of tissues exposed to oxidative DNA damage and found that folate depletion prevented induction of the BER pathway [20]. Further, we have shown that DNA strand breaks that arise in response to folate depletion accumulate to a larger degree when the BER pathway is genetically altered to have 50% reduction in capacity [21], demonstrating a direct role for the BER pathway in the DNA damage phenotypes of folate depletion.

Folate deficiency has been shown to result in an accumulation of uracil in DNA, a BER substrate, likely through altered thymidylate synthesis and a resulting dUMP/TMP imbalance. Uracil is uniquely removed from DNA by the BER pathway in a DNA-polymerase-β-(β-pol-) dependent fashion. In response to uracil accumulation, BER is initiated by a uracil DNA glycosylase (predominantly Udg). The processing of uracil induces transient DNA strand breaks that are ultimately resolved as repair is completed, as described in Figure 1. As such, if folate deficiency inhibits the BER pathway specifically as we have shown, we should then expect the folate deficient phenotype to mimic that of BER deficiency. In addition to accumulating DNA single-strand breaks, mutation frequencies, and DNA damage sensitivity described above, these folate-specific phenotypes also include chromosome breakage, micronucleus formation, defects in chromosomal condensation, and expression of chromosomal fragile sites ([22–26]. Many of these same phenotypes are induced by deficient BER such that the phenotypes of folate depletion closely mimic the phenotypes of BER deficiency. In Table 1 we present the phenotypes expressed by BER mutants. These phenotypes include uracil accumulation; mutation induction, increased DNA base damage, increased levels of DNA single and double strand

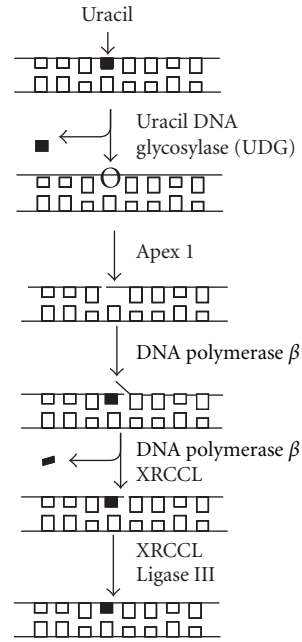

Figure 1: Biochemistry of base excision repair in uracil removal. Uracil removal is carried out as depicted, with initiation of removal by a uracil-excising DNA glycosylase (UDG depicted). All the uracil-excising glycosylases are monofunctional and leave behind an abasic lesion with an intact DNA backbone. An endonuclease (Apex 1) incises the DNA backbone 5′ to the abasic lesion, generating a 3′hydroxyl group and a 5′deoxyribose flap. A DNA polymerase (DNA polymerase β) inserts the correct nucleotide, then, in conjunction with a scaffolding protein (XRCCI), excises the deoxyribose flap. This step represents the rate-determining step in uracil-initiated base excision repair. Ligation of the scission in the phosphodiester backbone (ligase III and Xrcc1) completes repair and restores intact DNA structure. The single-strand break induced by Apex1 persists until ligation is complete and presents a potentially cytotoxic lesion if left incompletely repaired.

Table 1: Genome instability phenotypes in base excision repair mutant models.

Gene	Genotype	Phenotype	Genome instability
UNG [27–29]	$Ung^{-/-}$	Viable B-cell lymphoma Neuronal sensitivity to oxidative damage Neurodegeneration	Uracil accumulation in brain
SMUG [30, 31]	$Smug^{tg/+}$ $Smug^{/siRNA}Ung^{-/-}$	Viable	C to T mutagenesis
OGG1 [32–35]	$Ogg1^{-/-}$	Viable UVB-induced skin tumors	8-OHdG accumulation G to T mutagenesis Gamma radiation-induced DSB in OGG1 overexpressing cells
MYH [36, 37]	$Myh^{-/-}$ $Ogg1^{-/-}Myh^{-/-}$	Viable Reduced survival Lung, ovarian, and lymphoid tumors	Spontaneous mutagenesis G to T mutagenesis
AAG [38–40]	$Aag^{-/-}$	Viable Sensitivity to alkylation damage Retinal degeneration in +/−	Increased mutagenesis
NTH			
[41, 42] [35, 43]	$Nth1^{-/-}$ $Ogg1^{-/-}Nth^{-/-}$ TK6 cell line	Viable	Increased thymine glycol in liver after X-ray irradiation
[44]	$Ogg1^{-/-}Nth^{-/-}$ mice	Viable	Gamma irradiation-induced DSB H_2O_2 resistant
TDG [45]	$Tdg^{-/-}$	Embryonic lethal	Deficient repair of mtDNA
MBD4 [46, 47]	$Mbd4^{-/-}$ $Mbd4^{-/-}Apc^{min/+}$	Viable Intestinal adenomas	Aberrant chromatin metabolism C to T mutagenesis
FEN [48, 49]	$Fen1^{-/-}$ $Fen^{+/-}Apc^{1638N}$	Early embryonic lethal Intestinal adenocarcinoma Decreased survival	Microsatellite instability Extensive apoptosis
APE [50, 51] [20]	$Ape^{-/-}$ $Ape^{+/-}$	Embryonic lethal Sensitive to oxidative stress	
[52]	$Apex1^{+/-}XPC^{-/-}$	Increased UV-induced skin cancer	Increased mutagenicity Papillary adenocarcinoma and lymphoma
XRCC [53, 54]	$Xrcc1^{-/-}$	Embryonic lethal	
[55, 56]	$Xrcc1^{+/-}$	Increased AOM-induced ACF	SCE in embryo and cell lines
β -Pol [21, 64]	β-$Pol^{-/-}$	Embryonic lethal	DSB accumulation Increased mutagenesis
[57–60]	β-$Pol^{+/-}$	Viable Accelerated aging Lymphoma and adenocarcinomas	Increased SCE in MEFs SSB accumulation
LIGI [61]	$Lig^{-/-}$	Embryonic lethal defective erythropoiesis	Oxidative stress sensitivity Increased mutagenesis Chromosomal aberrations
LIGII [62]	$Lig^{-/-}$	Embryonic lethal	Elevated SCE

breaks, microsatellite instability, and increased levels of sister chromatid exchange (SCE) and chromosomal aberrations (referenced in Table 1 [20, 21, 27–62]). Our observation that folate depletion induced a phenotype very similar to that of BER depletion suggested to us that folate depletion might exert an inhibitory effect on activity of the BER pathway.

Accordingly, we have recently shown that the inhibitory effect of folate depletion on BER is achieved in part through inhibiting transactivation of the rate-limiting activity of BER, β-pol. Early work on the β-pol promoter clearly identified the CRE palindrome as being essential for ATF/CREB activation of the promoter [63]. We have identified a region within the β-pol CRE element that is blocked when folate is

depleted, and prevents transactivation when folate is deficient [20]. Demonstrating direct inhibition of the BER response to DNA damage is important with respect to connecting the phenotypes of folate depletion and BER deficiency. We suggest that the BER inhibition of folate depletion is dependent on initiation of the BER response without completion of repair, resulting in a repair imbalance. This comes from reports that the clastogenic phenotypes of BER deficiency are wholly dependent on glycosylase-mediated initiation of BER [64].

2.1. Uracil as a Source of Imbalanced Base Excision Repair and Double-Strand Break Formation. Glycosylase-mediated induction of BER begins a series of enzymatic reactions that induces a break in the phosphodiester backbone; a break that persists until repair is completed (Figure 1). We have shown that folate depletion induces uracil DNA glycosylase (UDG) activity in liver without a corresponding induction of the rest of the pathway, generating an imbalance in BER and an accumulation of DNA single-strand breaks [21]. Others have likewise shown accumulation of single strand breaks and double strand breaks in response to folate depletion [65, 66]. Strand breaks set the stage for chromosomal instability including dicentric formation, anaphase bridges, and gross amplifications/deletions [67]. We propose that it is through these uracil-initiated strand breaks and chromosomal aberrations that folate promotes micronuclei formation, a likely carcinogenic precursor. Recently, MacFarlane et al. have demonstrated a key role for uracil misincorporation as a driving force in the colorectal carcinogenesis of folate depletion ([68]). Folate depletion and serine hydroxymethyltransferase heterozygosity ($SHMT^{+/-}$) resulted in a twofold increase in number of colon tumors in the APCmin model of intestinal tumorigenesis. Moreover colonic DNA uracil content doubled when $APC^{min/+}$ $SHMT^{-/+}$ mice were folate depleted, correlating to observed decreases in thymidylate synthesis protein abundance. SHMT uses serine as the one carbon donor to convert tetrahydrofolate (THF) to 5,10 methyleneTHF, the 1C donor for conversion of dUMP to TMP. These results directly connect the increased tumorigenesis observed in colons of $APC^{min/+}$ $SHMT^{-/+}$ to changes in thymidylate synthesis and resultant uracil misincorporation into DNA.

3. Folate Depletion and Colorectal Carcinogenesis

Many epidemiological studies support the protective effect of folate in prevention of colorectal cancer. Most recently, a meta-analysis of 13 human studies shows a positive correlation between folate consumption and protection from colorectal cancer [69]. Accordingly, many rodent studies demonstrate that folate depletion increases tumorigenesis and/or the development of precursor lesions (aberrant crypt foci, ACF) in response to colon carcinogens. In the past decade, this protective effect of folate supplementation and detrimental effect of folate depletion have been called into question in response to several studies in which folate supplementation increased the numbers of tumors in tumor models of colorectal carcinogenesis, and correspondingly folate depletion reduced tumor development or aberrant crypt formation. These contradictory findings have been reviewed [70], and the short explanation is that folate supplementation is potentially detrimental during the promotion phase of carcinogenesis, either as a function of carcinogen exposure or genetic manipulation.

3.1. Analysis of Dietary Intervention Strategies and Impact of Folate Status and Intestinal Endpoints. Conclusions about the potential dangers of folate on colorectal cancer development may be based, in some instances, on unequal comparisons. A primary objective of this paper is to complete a careful analysis of dietary intervention studies to evaluate the importance that differences in model systems and/or dietary interventions may have on critical colorectal cancer endpoints. In Table 2, we have tabulated this information to allow facile comparison of dietary interventions across studies. We have included information on the following: animal model, diet source (when provided), length of dietary intervention, whether the study design included use of antibiotics to prevent microbial production of folates in the colon or use of wire-bottom caging to prevent coprophagy, and, the impact of the dietary intervention on folate levels (when provided). Included are studies that present data pertaining specifically to colorectal cancer endpoints, including mutation frequency, ACF, and intestinal tumors [71–81]. (These endpoints are presented in detail in Table 4). What we find is that the typical dietary intervention uses a dietary prescription of 2 mg/kg folate for the control group, with experimental groups of 0 mg/kg folate (deficient) and 8 mg/kg (supplemented). Some studies have used 20 mg/kg folate as a hypersupplemented group as well, which does not appear to confer additional advantage or disadvantage. Some studies have used 8 mg/kg folate as the control diet [73] and some have used 5 mg/kg folate as the control diet [82], which makes comparison across studies difficult. As standard chow diets provide on average 8–10 mg/kg folic acid, these study designs facilitate comparisons across studies in which undefined diets have been used. However, it does make interpretation of the data difficult within the context of folate dose on cancer risk. This is illustrated in the summary data we present (Table 3) on percent change in blood folate and colonic folate status in response to dietary intervention. Using 2 mg/kg folate as control, there is a 50–96% decrease in blood folate at 0 mg/kg folate (with the use of antibiotics or prevention of coprophagy), and a 58–140% increase at 8 mg/kg folate. This makes it difficult to interpret the role of folate depletion and/or supplementation when the comparison is between 8 mg/kg and 0 mg/kg folate.

Duration of dietary intervention appears to affect the impact on folate status. Very few studies have measured colonic folate status in response to dietary depletion, which is the target tissue of interest for this paper. But in two papers in which this was determined there seems to be a significant impact of increasing the length of the study on colonic folate levels. After 8 weeks of feeding, rats exhibit

Table 2: Impact of experimental design on blood and tissue folate.

Animal model	Experimental diet	Abx	Wire cages	Length of feeding	In vivo folate levels	Citation
Rat studies quantifying impact of dietary intervention on blood and/or tissue folate status						
Sprague-Dawley rats	Amino acid defined (Dyets) *Control group*: 8 mg/kg folate *Experimental group*: 0 mg/kg folate	No	Yes	25 weeks	Folate levels (nmol/g) *Control group:* Liver 27.11 Kidney 11.69 Spleen 3.74 Brain 0.65 *Experimental group:* Liver 11.10 Kidney 4.79 Spleen 1.29 Brain 0.60	[71]
Sprague-Dawley rats	Amino acid defined (Dyets) *Control group*: 2 mg/kg folate *Experimental groups*: 0 mg/kg folate 8 mg/kg folate 20 mg/kg folate	No	Yes	8 weeks	*Control group*: Plasma folate (ng/mL) ~50 Colonic folate (ng/mL) ~650 *Experimental groups*: Deficient Plasma folate (ng/mL) ~25 Colonic folate (ng/mL) ~480 Supplemented (8 mg/kg) Plasma folate (ng/mL) ~80 Colonic folate (ng/mL) ~975 Supplemented (20 mg/kg) Plasma folate (ng/mL) ~140 Colonic folate (ng/mL) ~975	[83]
Sprague-Dawley rats	AIN-76 semipurified diet *Control group:* 8 mg/kg folate, no abx *Experimental groups:* 0 mg/kg folate, no abx 0 mg/kg folate, with abx first 4 weeks of feeding 0 mg/kg folate, with abx last 4 weeks of feeding	Yes and no	Yes	12 weeks	Whole blood folate *Control group:* 657 ng/mL *Experimental group:* 125 ng/mL (no abx) 114 ng/mL (abx first 4 weeks) 61 ng/mL (abx last 4 weeks)	[72]
Sprague-Dawley rats	AIN-76 semipurified diet *Control group:* 8 mg/kg folate, no abx *Experimental groups:* 0 mg/kg folate, no abx 0 mg/kg folate, with abx first 4 weeks of feeding 0 mg/kg folate, with abx 4 weeks after AOM	Yes and no	Yes	26 weeks	Whole blood folate *Control group:* 684 ng/mL *Experimental groups:* 694 ng/mL (control + abx) 99 ng/mL (0 mg/kg, no abx) 100 ng/mL (0 mg/kg, abx first 4 weeks) 96 ng/mL (0 mg/kg, abx 4 weeks after AOM) Colon folate *Control group:* 9.6 ug/mg *Experimental groups:* 8.0 ug/mg (control + abx) 2.7 ug/mg (0 mg/kg, no abx) 3.1 ug/mg (0 mg/kg, abx first 4 weeks) 4.3 ug/mg (0 mg/kg, abx 4 weeks after AOM)	[73]

Table 2: Continued.

Animal model	Experimental diet	Abx	Wire cages	Length of feeding	In vivo folate levels	Citation
Sprague-Dawley rats	AIN93 (G or M not Specified) *Control group:* 2 mg/kg folate *Experimental group:* 0 mg/kg folate	No	No	20 weeks	Hepatic folate (nmol/g) *Control group:* 17 nmol/g (weanling) 17 nmol/g (12 month old) *Experimental group:* 2.7 nmol/g (weanling) 1.8 nmol/g (12 month old)	[74]
Sprague Dawley rats	AIN93 purified diet (G or M not specified) *Control group* 2 mg/kg (folate-replete) *Experimental groups:* 0 mg/kg folate 8 mg/kg	No	Yes	20 weeks	Plasma folate Young Old *Control group:* (umol/L) 34.1 30.6 *[(ng/mL) 15.1 13.5]* *Experimental group:* Deficient (umol/L) 1.5, 0.9 *[(ng/mL) 0.7 0.04]* *Supplemented:* (umol/L) 53.9 55.5 *[(ng/mL 23.8 24.6]* Colon (nmol/g) Young Old Control 4.7 3.0 Deficient 1.3 0.7 Supplemented 5.6 4.4	[75]
Fischer-344 rats	AIN-93 diet *Control group:* 2 mg/kg folate *Experimental group:* 0 mg/kg folate Note: selenium assessed in this paper, not evaluated here.	No	Yes	11 weeks	Plasma folate *Control group:* 277.4 nmol/L *[122.44 ng/mL]* *Experimental group:* 12.0 nmol/L *[5.30 ng/mL]*	[85]
Sprague-Dawley rats	Amino acid defined (Dyets) All animals fed same diet (2 mg/kg folate) for first 10 weeks; 5 weeks following completion of carcinogen exposure, experimental diets began. *Control group:* 2 mg/kg folate *Experimental groups:* 0 mg/kg folate 5 mg/kg folate 8 mg/kg folate	Yes	No	24 weeks	Plasma folate *Control group:* 32.2 ng/mL *Experimental groups:* Deficient 6.0 ng/mL Supplemented (5 mg/kg) 72.8 ng/mL Supplemented (8 mg/kg) 78.2 ng/mL Hepatic folate *Control group:* 7.7 ug/g *Experimental groups:* Deficient 5.0 ug/g Supplemented (5 mg/kg) 8.5 ug/g Supplemented (8 mg/kg) 9.9 ug/g	[76]

Table 2: Continued.

Animal model	Experimental diet	Abx	Wire cages	Length of feeding	In vivo folate levels	Citation
Male Hooded-Lister rats	AIN-93G purified diet with vitamin-free casein *Control group:* 5 mg/kg folate *Experimental group:* 0 mg/kg folate	No	Yes	6 weeks	Folate value, ng/mg protein Lymphocytes Control: 0.45 Experimental: 0.27 Liver Control: 136.2 Experimental: 93.9 Colon (descending) Control: 24.6 Experimental: 9.9 Spleen Control: 16.5 Experimental: 7.9 Kidney Control: 58.6 Experimental: 26.6 Brain Control: 20.4 Experimental: 15.3 Heart Control: 9.2 Experimental: 3.4	[82]
Mouse studies quantifying impact of dietary intervention on blood and/or tissue folate status						
C57bl/6J mice, APC^{Min}	Amino acid defined (Dyets) *Control group:* 2 mg/kg folate *Experimental group:* 0 mg/kg folate 8 mg/kg folate* 20 mg/kg folate	No	No	3 months; 6 months	Serum folate (ng/mL): *Control group:* 39.0 (3 months) 35.4 (6 months) *Experimental group:* Depleted 12.1 (3 months) 10.8 (6 months) Supplemented 56.0 (3 months) 46.0 (6 months) Hypersupplemented 49.9 (3 months) 43.3 (6 months)	[77]
C57bl/6 mice, β-$pol^{+/-}$	AIN-93G purified diet with vitamin-free casein *Control group:* 2 mg/kg folate *Experimental group:* 0 mg/kg folate	Yes	No	8 weeks	Serum folate *Control group:* 60 ng/mL *Experimental group:* <5 ng/mL	[21]
C57bl/6 mice, $Aag^{-/-}$	AIN-93G purified diet with vitamin-free casein *Control group:* 2 mg/kg folate *Experimental group:* 0 mg/kg folate	Yes	Yes	4 weeks	Liver folate *Control group* 27.0 μg/g *Experimental group* 1.2 μg/g	[39]
					$Apc^{+/+}$ crossed to SHMT genotype as indicated $^{(+/+,+/-,-/-)}$ *Control group: (5 weeks)* Plasma (ng/mL) 58.56 (+/+) 58.34 (+/−) 40.82 (−/−) Liver (fmol/ug pro) 51.80 (+/+) 56.65 (+/−) 50.77 (−/−)	

Table 2: Continued.

Animal model	Experimental diet	Abx	Wire cages	Length of feeding	In vivo folate levels	Citation
C57bl/6 mice, *Apc*$^{min/+}$ Shmt1 ($^{+/-}$ and $^{-/-}$)	AIN-93G purified diet *Control group:* 2 mg/kg folate 2.5 g/kg choline *Experimental group:* 0 mg/kg folate 0 g/kg choline	No	Yes	5 weeks (Apc$^{+/+}$); 11 weeks (Apc$^{min/+}$)	Colon (fmol/ugpro) 35.14 (+/+) 21.46 (+/−) 17.09 (−/−) *Experimental group: (5 weeks)* Plasma (ng/mL) 20.60 (+/+) 38.95 (+/−) 8.52 (−/−) Liver (fmol/ug pro) 47.26 (+/+) 44.30 (+/−) 48.88 (−/−) Colon (fmol/ugpro) 9.15 (+/+) 18.04 (+/−) 14.89 (−/−) *Apc*$^{min/+}$ *crossed to Shmt genotype indicated* ($^{+/+,+/-,-/-}$) *Control group: (11 weeks)* Plasma (ng/mL) 24.68 (+/+) 20.91 (+/−) 26.44 (−/−) Liver (fmol/ug pro) 45.72 (+/+) 40.00 (+/−) 41.50 (−/−) *Experimental group: (11 weeks)* Plasma (ng/mL) 11.79 (+/+) 8.37 (+/−) 9.97 (−/−) Liver (fmol/ug pro) 28.49 (+/+) 23.25 (+/−) 29.44 (−/−)	[68]
C57bl/6J mice, Shmt ($^{+/-}$ and $^{-/-}$)	AIN-93G purified diet *Control group:* 2 mg/kg folate 2.5 g/kg choline *Experimental group:* 0 mg/kg folate 0 g/kg choline	No	No	32 weeks	*Control group:* Plasma folate (ng/mL) (wt) 36.3 (Tg) 46.8 Liver folate (fmol/ug pro) (wt) 43.1 (Tg) 51.3 *Experimental group:* Plasma folate (ng/mL) (wt) 7.4 (Tg) 5.5 Liver folate (fmol/ug pro) (wt) 36.1 (Tg) 34.0	[68]
C57bl/6 mice, APC^{1638N}	Amino acid defined (Dyets) *Control group:* 2 mg/kg folate B-vitamin adequate *Experimental group:* 0 mg/kg folate B12, B6, and riboflavin deficient	No	No	16 weeks	*Control group:* Plasma folate (ng/mL) ~170 Colon folate (ng/g) ~500 *Experimental group:* Plasma folate (ng/mL) ~110 Colon folate (ng/g) ~300	[84]

Table 2: Continued.

Animal model	Experimental diet	Abx	Wire cages	Length of feeding	In vivo folate levels	Citation
C57bl/6 mice, APC^{1638N}	*Maternal diet:* AIN93M (Dyets) Control and experimental *Offspring diet:* AIN93G during first 16 weeks of life AIN93M during last 16 weeks of life (Dyets) Control and experimental *Control group:* 2 mg/kg folate 6 mg/kg riboflavin 7 mg/kg B6 50 ug/kg B12 *Experimental groups:* Deficient 0.5 mg/kg folate 2 mg/kg riboflavin 2 mg/kg B6 10 ug/kg B12 Supplemented 8 mg/kg folate 24 mg/kg riboflavin 28 mg/kg B6 200 ug/kg B12	No	No	*Maternal diet:* Fed 4 weeks preconception through weaning *Offspring diet:* 32 weeks	Maternal *Control group:* Plasma folate (ng/mL) 84.7 Hepatic folate (ug/g) 13.2 *Experimental group:* Deficient Plasma folate (ng/mL) 81.4 Hepatic folate (ug/g) 11.1 Supplemented Plasma folate (ng/mL) 104.4 Hepatic folate (ug/g) 13.3 Offspring *Control group:* Plasma folate (ng/mL) 52.5 Hepatic folate (ug/g) 14.1 Sm Int folate (ng/g) 1205.9 *Experimental group:* Deficient Plasma folate (ng/mL) 59.3 Hepatic folate (ug/g) 12.9 Sm Int folate (ng/g) 1264.7 Supplemented Plasma folate (ng/mL) 50.2 Hepatic folate (ug/g) 13.3 Sm Int folate (ng/g) 1172.4	[78]
Folate depletion studies presenting critical colorectal cancer endpoints, but without folate status information						
Fisher 344 rats	AIN93G *Control group:* 2 mg/kg folate, −abx *Experimental groups:* 0 mg/kg folate, −abx 0 mg/kg folate, +abx	Yes and no	No	5 weeks	ND	[15]
Fisher 344 rats	NIH-31 *Control group:* 0.4% methionine 0.3% choline 2 mg/kg folate *Experimental group:* Low methionine 0% choline 0 mg/kg folate	No	No	36 weeks 54 weeks	ND	[79]
C57bl/6J mice	Casein/soy based *Control group:* 2 mg/kg folate *Experimental group:* 0 mg/kg folate	No	No	10 weeks	ND	[80]
BALB/cAnNCrlBR mice	Amino acid defined (Harlan Teklad) *Control group:* 2 mg/kg folate *Experimental group:* 0.3 mg/kg folate	Yes	No	12 to 14 months	ND	[81]
C57bl/6 mice $Bpol^{+/-}$	AIN93G (Dyets) *Control group:* 2 mg/kg folate *Experimental group:* 0 mg/kg folate	Yes	No	12 weeks total (6 pre-DMH; 6 post-DMH)	ND	[59]

Table 2: Continued.

Animal model	Experimental diet	Abx	Wire cages	Length of feeding	In vivo folate levels	Citation
Albino rats	AIN93M *Control group:* 2 mg/kg folate *Experimental groups:* 8 mg/kg folate 40 mg/kg folate	No	No	6 weeks total (4 weeks pre-AOM; 2 weeks post-AOM)	ND	[86]

Values in brackets [] have been calculated from published values for ease of comparison across studies; +/+, +/− and −/− refer to wildtype, heterozygous and null genotypes; ND: not determined.

Table 3: Impact of dietary intervention on blood and colon folate status.

Percent change in blood folate status by dietary intervention			
2 mg/kg to 0 mg/kg	↓96%	Rat	20 wk
(with either abx or wire bottom cages)	↓96%	Rat	11 wk
	↓92%	Mouse	8 wk
[21, 75, 76, 83, 85]	↓50%	Rat	8 wk
	↓81%	Rat	24 wk
2 mg/kg to 0 mg/kg	↓69%	Mouse	12 wk
(without abx or wire bottom cages)	↓63%*	Mouse	5 wk
	↓78%*	Mouse	11 wk
[68, 77, 84]	↓35%**	Mouse	16 wk
2 mg/kg to 8 mg/kg	↑58%	Rat	20 wk
(with either abx or wire bottom cages)	↑62%	Rat	8 wk
[75, 76, 83]	↑140%	Rat	24 wk
2 mg/kg to 8 mg/kg (without abx or wire bottom cages) [77]	↑44%	Mouse	12 wk
Percent change in colon folate status by dietary intervention			
2 mg/kg to 0 mg/kg	↓72%	Rat	20 wk
(with either abx or wire bottom cages) [75, 83]	↓35%	Rat	8 wk
2 mg/kg to 0 mg/kg	↓74%*	Mouse	5 wk
(without abx or wire bottom cages) [68, 84]	↓40%**	Mouse	16 wk
2 mg/kg to 8 mg/kg	↑19%	Rat	20 wk
(with either abx or wire bottom cages) [75, 83]	↑66%	Rat	8 wk
2 mg/kg to 8 mg/kg (without abx or wire bottom cages)			

Abx: antibiotics; wk: week; *choline also depleted in this dietary intervention; **riboflavin, B6, and B12 also modified in this dietary intervention.

a 35% decrease in colon folate levels [83], but after 20 weeks there is a 72% decline [75]. The data in mice seems odd, in that the colon folate levels are more depleted after 5 weeks of dietary intervention [68] than after 16 weeks [84], but these studies are confounded by alterations in choline and other B vitamins. Further, these mouse studies were carried out in the absence of methods for reducing assimilation of microbial-produced folates in the colon, and there is a possibility that with the extended intervention time (16 weeks) animals adapt to reduced dietary availability of nutrients through increased coprophagy.

There also seems to be a differential sensitivity to folate depletion between mice and rats. From the limited data available, mice appear to become severely depleted (>90% reduced blood folate) after 8 weeks of feeding [21], while rats are only about 50% depleted at 8 weeks [83], 1996, but >90% depleted by 11 weeks or more of dietary folate restriction [85]. This holds true for colonic folate status as well. However, whether this 3-week difference is meaningful is unclear.

The information in Table 3 clearly demonstrates the importance of antibiotic and/or wire-bottom cages for inducing the most severe folate depletion. The average reduction in blood folate when antibiotics and/or wire-bottom cages are used is >80% (with the majority of studies showing >90%), while the absence of these factors induces a more modest 61% reduction. Further evidence for the importance of severe dietary restrictions to induce a meaningful decline in folate status is the finding that 32 weeks of feeding 0.5 mg/kg folate without antibiotics or wire-bottom cages resulted in no change in blood folate status [78]. It would be useful to have data on the impact of folate depletion on colonic folate status with and without contribution from microbially produced folates, but that information is not available without the confounding of choline deficiency [68] or riboflavin, B6, and B12 deficiencies [84]. A systematic evaluation of dietary factors on target tissue folate status would be informative.

3.2. Impact of Dietary Folate Restriction on Colorectal Cancer Endpoints. These considerations aside, there is a definite impact of altering blood and tissue folate status on colorectal cancer endpoints. And these differences seem to be clearly dependent on the stage of cancer development. Table 4 outlines studies in which colorectal cancer endpoints have been analyzed in response to dietary folate manipulations. It is very clear that in rats exposed to DMH (dimethylhydrazine), ENU (ethyl nitroso-urea), or AOM (azoxymethane) that dietary folate depletion is detrimental if the diet is begun prior to carcinogen exposure. Rats exposed to DMH had a 70% increase in colon tumors [83] and a 66% increase in ACF [85] when folate was depleted in the diet. In response to AOM, the increase in aberrant crypt foci was more modest at 12% increase [86], likely a function of

Table 4: Impact of experimental design on critical colorectal cancer endpoints.

Animal model	Carcinogen	CRC-specific endpoints measured
Studies demonstrating beneficial effects of folate on critical colorectal cancer endpoints		
Rat Sprague-Dawley Male [83]	DMH 44 mg/kg body weight Weekly × 15 weeks	*Percent of rats with colonic tumors*: 70% (0 mg/kg folate) **40% (2 mg/kg folate)** 10% (8 mg/kg folate) 42% (40 mg/kg folate) (Similar results for # tumors/rat)
Rat Fisher 344 Male [15]	5-week diet prior to DMH ENU 100mg/kg	*Mutant frequency*: 8-fold increase (0 mg/kg folate, no antibiotics) 6-fold increase (0 mg/kg folate, with antibiotics) **5-fold increase (2 mg/kg folate)**
Rat Fisher 344 Male [85]	DMH 25 mg/kg body weight 2 weekly injections	*Colonic aberrant crypts/aberrant crypt foci*: **~150 aberrant crypts, 50 foci (2 mg/kg folate)** ~250 aberrant crypts, 75 foci (0 mg/kg folate)
Rat Albino Male [86]	3-week diet prior to DMH 8-week diet after DMH AOM 30 mg/kg body weight	*Aberrant crypt foci*: **~65 aberrant crypts (2 mg/kg folate)** ~58 aberrant crypts (8 mg/kg folate) ~30 aberrant crypts (40 mg/kg folate) (note: no deficient group)
Mice Balb/cAnNCrlBR Wildtype (from 129) Backcrossed >10 generations into Balb/c Sex: not stated [81]	None, diet only	*Percent mice with duodenal tumors*: **0% (2 mg/gk folate)** 12.5% (0.3 mg/kg folate) (2/16 mice; adenoma versus adenocarcinoma not specified)
Mice C57bl/6 Apc1638N, BAT-LacZ (Wnt reporter mouse) No. of generations backcrossed N/A Sex: not stated [84]	None, diet and genotype only (Note: diet includes multiple B vitamin manipulations: Riboflavin, B6, B12, and folate)	*Gastrointestinal tumor incidence and multiplicity; aberrant crypt foci*: **50% incidence (2 mg/kg folate, adequate Bvitamins)** 91% incidence (0 mg/kg folate, B vitamin deficient) **~1.7 tumors/animal (2 mg/kg folate, adequate Bvitamins)** ~2.7 tumors/animal (0 mg/kg folate, B vitamin deficient) **~2 aberrant crypts (2 mg/kg folate, adequate Bvitamins)** ~2.5 aberrant crypts (0 mg/kg folate, B vitamin deficient)
Mice C57bl/6 Apc1638N No. of generations backcrossed N/A Sex: both [78]	None, diet and genotype only (Note: diet includes multiple B vitamin manipulations: Riboflavin, B6, B12, and folate; dietary intervention in dams and offspring)	*Gastrointestinal tumor incidence (percent) and multiplicity*: ~55% incidence (0.5 mg/kg folate, B vitamin deficient) **~58% incidence (2.0 mg/kg folate, Bvitamin adequate)** ~20% incidence (8.0 mg/kg folate, B vitamin supplemented) ~0.6 tumors/animal (0.5 mg/kg folate, B vitamin deficient) **~0.6 tumors/animal (2.0 mg/kg folate, Bvitamin adequate)** ~0.25 tumors/animal (8.0 mg/kg folate, B vitamin supplemented) (Note: tumor invasiveness significantly worse in deficient group compared to control group)
Mice C57bl/6 Apc^{min} >10 generations backcrossed Sex: not stated [68]	None, diet and genotype only Shmt heterozygous and null genotypes crossed onto Apc^{min} Note: choline altered as well as folate	*Gastrointestinal tumor number and tumor load (total tumor area/mouse)*: Impact of diet seen only in Shmt heterozygous model: **~32 small intestinal tumors (2 mg/kg folate, 2.5 g/kg choline)** ~60 small intestinal tumors (0 mg/kg folate, 0 g/kg choline) **~40 mm^2 tumor load (2 mg/kg folate, 2.5 g/kg choline)** ~80 mm^2 tumor load (0 mg/kg folate, 0 g/kg choline)
Mice C57bl/6 DNA polymerase $\beta^{+/-}$ >10 generations Sex: Male [59]	DMH 30 mg/kg body weight Weekly for 6 weeks Killed after 12 weeks	*Total aberrant crypt foci*: **~15 aberrant crypts (2 mg/kg folate, wildtype)** ~38 aberrant crypts (0 mg/kg folate, wildtype) (Note: this work presents data both in support of protective and detrimental roles for folate; protective presented here, detrimental presented below)

Table 4: Continued.

Animal model	Carcinogen	CRC-specific endpoints measured
Rat Sprague-Dawley Male [72]	AOM 15 mg/kg Weekly for 3 weeks Killed after 8 weeks	*Total aberrant crypt foci*: ~300 aberrant crypts (8 mg/kg folate) ~200 aberrant crypts (0 mg/kg folate + abx post-AOM) (Note: no "standard" control group of 2 mg/kg folate)
Rat Sprague-Dawley Male [73]	AOM 15 mg/kg/week Weekly for 3 weeks Killed after 22 weeks	*Number of colon adenocarcinomas*: 13 (8 mg/kg folate, no abx) 12 (8 mg/kg folate, with abx) 4 (0 mg/kg folate, no abx) 3 (0 mg/kg folate, abx before AOM) 4 (0 mg/kg folate, abx after AOM) (Note: no "standard" control group of 2 mg/kg folate)
Rat Sprague-Dawley Male [76]	AOM 2 weekly exposures (total dosing unclear) Diet begun 6 weeks post-AOM	*Aberrant crypt foci and tumor size (tumor diameter/tumor-bearing animal, cm)* 84.6 aberrant crypts (0 mg/kg folate) **93.4 aberrant crypts (2 mg/kg folate)** 108.1 aberrant crypts (5 mg/kg folate) 137.9 aberrant crypts (8 mg/kg folate) 0.5 cm (0 mg/kg folate) **1.2 cm (2 mg/kg folate)** 1.3 cm (5 mg/kg folate) 1.6 cm (8 mg/kg folate)
Mice C57bl/6 APC^{min} No. of generations backcrossed N/A Sex: not stated [77]	None, diet and genotype only Two timepoints: 3 and 6 months	*Aberrant crypt foci and ileal adenomas*: At 3 months: 1.3 aberrant crypts (0 mg/kg folate) **0.27 aberrant crypts (2 mg/kg/folate)** 0.20 aberrant crypts (8 mg/kg folate) 0.00 aberrant crypts (20 mg/kg folate) 11.0 ileal adenomas (0 mg/kg folate) **7.36 ileal adenomas (2 mg/kg folate)** 7.30 ileal adenomas (8 mg/kg folate) 2.36 ileal adenomas (20 mg/kg folate) At 6 months: 1.67 ileal adenomas (0 mg/kg folate) **7.09 ileal adenomas (2 mg/kg folate)** 5.33 ileal adenomas (8 mg/kg folate) 4.38 ileal adenomas (20 mg/kg folate)
Mice C57bl/6 DNA polymerase $\beta^{+/-}$ >10 generations Sex: male [59]	DMH 30 mg/kg body weight Weekly for 6 weeks Killed after 12 weeks	*Total aberrant crypt foci:* **~33 aberrant crypts (2 mg/kg folate, heterozygote)** ~20 aberrant crypts (0 mg/kg folate, heterozygote) (Note: this work presents data both in support of protective and detrimental roles for folate; detrimental presented here, protective presented above.)

~indicates values are approximated from graphical data; N/A: not available; Shmt: serine hydroxyl methyl transferase.

a less restrictive dietary intervention (no antibiotics or wire-bottom caging). Because colorectal cancer is believed to be initiated by mutations in key genes, we also analyzed a paper in which the mutagenic response to ENU was evaluated (not a colon carcinogen) and found that ENU increased mutant frequency 8-fold when folate was depleted [15]. However, a very different effect is seen when diet is begun after carcinogen exposure. With respect to ACF and tumor formation, when folate depletion began post-AOM exposure, a slight decrease in ACF and tumor diameter was seen in the folate depleted group (0 mg/kg foalte) and a 47% increase was seen in the folate supplemented group (8 mg/kg folate) [76], suggesting that presence of folate was permissive for ACF and tumor formation. With respect to colon adenocarcinomas, Le Leu et al. [73] found that the total number of adenocarcinomas was 3-fold higher in the 8 mg/kg folate group as compared to the 0 mg/kg group. Clearly in this example folate depletion was protective against AOM-induced colon adenocarcinomas and folate supplementation was detrimental, but it remains unclear how either of these groups would have compared to an adequate folate diet of 2 mg/kg folate.

In mouse studies, data are confounded by genotype differences in models predisposed to develop gastrointestinal tumors, as well as other genetic manipulations devised to investigate the role(s) of certain pathways on colon

tumorigenesis. In total we present 4 mouse studies showing a protective effect of folate on colon tumorigenesis, and 2 studies showing both detrimental and protective effects. Each study presents its own limitations preventing direct comparisons and solid conclusions. For example, in the APC^{min} mouse, two studies have been completed that reach two different conclusions. In the MacFarlane et al. study [68], three *Shmt* genotypes were investigated, wildtype, heterozygous, and null mutants. The authors found an effect of diet on tumor number and size in the *Shmt* heterozygous mice only, showing a 2-fold increase in both variables when folate was deficient. However, in this study choline was also depleted, making it difficult to know the specific impact of folate on tumorigenesis. In contrast, Song et al. [77] found that at 3 months folate depletion was detrimental (more ACF and more ileal adenomas as compared to 2 mg/kg folate), but that by 6 months folate depletion was significantly protective against the development of ileal adenomas. Oddly, folate supplementation was also protective, with the 2 mg/kg folate group having the highest number of ileal adenomas. The protective effect of folate observed in the MacFarlane et al. was seen only in the *Shmt* heterozygous animals, which is to say that in the wildtype animals which provides the appropriate comparison to the Song et al. study, no effect of diet was seen on tumorigenesis. Of note, neither of these studies utilized either antibiotics or wire-bottom caging, such that the impact of the dietary intervention on tissue specific folate status was likely moderate.

In two studies using a different APC model, the APC1638N mouse, riboflavin, B6, and B12 deficiencies were investigated along with folate deficiency such that the conclusions are not specific to folate. Additionally, both these studies avoided use of antibiotics and wire-bottom caging, so the impact of dietary intervention on folate status was moderate (see Table 3). Interestingly, in the Liu et al. study, this moderate folate depletion resulted in an approximate 40% decline in colon folate status. The Ciappio study measured small intestinal folate levels and found no difference in response to dietary manipulation; findings that are somewhat counterintuitive. With respect to the critical endpoints, the Liu et al. study found an almost 2-fold increase in tumor incidence (50% compared to 91%) in response to folate (and other B vitamins) depletion. This corresponded to an increased number of tumors/animal and a slight increase in aberrant crypt numbers. Ciappio et al. investigated the impact of maternal diet on cancer in offspring. Mothers were depleted during pregnancy and weaning, then offspring were separated into vitamin B-sufficient-, deficient- and supplemented-groups. Notably, the folate level in the vitamin-B-deficient group was 0.5 mg/kg folate, which effectively resulted in no changes in serum or small intestinal folate. This suggests that changes observed in critical endpoints may not be due to folate status but rather to other B-vitamins. Nonetheless, they observe a strongly protective effect of B-vitamin supplementation on both tumor incidence and multiplicity (~60% reduction). Oddly, B-vitamin supplementation resulted in the lowest target tissue (small intestine) and blood folate levels making it difficult to interpret the data with respect to tissue folate status. However, it is noteworthy that the deficient group exhibited the worst tumor invasiveness of all groups, clearly an important endpoint.

In the only study to investigate tumorigenesis in response to folate depletion in a mouse strain other than C57bl/6, Knock et al. have shown that folate depletion increased the number of duodenal tumors in the BALB/c strain [81]. Unfortunately folate status was not determined, but the use of antibiotics suggests that these animals would have been significantly depleted. This was a relatively small number of animals (2/16 developed the duodenal tumors), but none of the mice in the control group developed tumors, so there is a definite effect in these animals as compared to the C57bl/6. The effect of strain differences in susceptibility to dietary intervention is potentially quite interesting. Studies have demonstrated significant copy number changes between strains, and even between substrains [87, 88], and these changes could potentially account for the observed differences. Along these lines, it's also important to consider the impact that multiple crossings into different strains (129 for making transgenics, e.g.) may have. Even with what is presumed to be adequate backcrossing, it's inevitable that some DNA sequence does not get fully backcrossed out. In this reviewer's opinion, this consideration has broad implications, but implications that also present opportunities for exploitation of copy number change-induced phenotype effects. To my knowledge, the only other study that has shown increased incidence of cancer in response to dietary folate manipulation was Pogribny and James [79], in which methyl donor deficiency resulted in liver tumors. To date, there are no studies showing that dietary folate (or methyl donor, or B-vitamin) deficiency induces colon tumors in rodents in the absence of carcinogen exposure. Even the genetic manipulations do not induce colon tumors, so data for answering this question is lacking.

Two studies present data demonstrating both protective and detrimental effects of folate on critical endpoints. Song et al. [77] described above, found that at 3 months of age in the APC^{min} mouse folate was protective, while at 6 months of age provision of folate was detrimental. These findings suggest that folate may help prevent initiation (3 months) while fueling tumor growth during promotion (6 months), consistent with the rat studies showing that folate drives tumorigenesis in the initiated colon. In the Ventrella-Lucente et al. paper [59], the impact of DNA base excision repair (BER) capacity on the tumorigenesis of folate depletion was investigated using a mouse model of BER deficiency [57]. This model develops lymphoma and adenocarcinoma in the absence of chemical exposure [58], suggesting that they would be more susceptible to the carcinogenic impact of folate deficiency. Using ACF as the critical endpoint, they demonstrated that folate depletion more than doubled the number of ACF in response to DMH exposure, consistent with the findings in rat models described above [83, 85]. However, in the BER mutant animals (DNA polymerase β heterozygotes), the reverse effect was seen on ACF formation. Here folate depletion significantly reduced the total number of ACF in response to DMH. DNA repair deficiencies are modifiers of penetrance [89], but typically in the opposite

direction observed in the Ventrella-Lucente paper. However, another study looking at the APC^{min} mouse likewise found that a DNA repair mutant (Ku70) ameliorated the impact of the APC mutation on tumorigenesis [90]. This study did not investigate a role for folate, but demonstrates the counterintuitive impact that loss of DNA repair capacity can have on tumorigenesis.

4. Conclusions

It becomes clear that while each study presents important information regarding the impact of folate on genomic stability in the colon, that there is some problem with a lack of consistency across study designs that prevent us from arriving at definitive conclusions. As the body of literature on folate continues to grow, these gaps in knowledge will be filled. We suggest that there is still an important need for a comprehensive study investigating the impact of differential folate prescriptions on blood and tissue folate status. We have shown here that the duration of feeding, dosage of folate, and use or avoidance of antibiotics and/or wire-bottom caging all impact the severity of folate depletion. Another point to consider is the difference in total blood folate levels between rodents and humans. The normal range for serum folate in humans is 2.7–17 ng/mL, manyfold lower than the average values observed in mice and rats. The range for mouse values reported in Table 2 is 39–170 ng/mL, for an average of 82.5 ng/mL, or about 8-fold higher than human values. The absolute values for rats reported in Table 2 range from 34 to 684 ng/mL. Excluding the two very high values (657 and 684 ng/mL), the average rat blood folate level is 60 ng/mL—6-fold greater than human. In essence, the dietary regimens that we consider to be folate depleted in rodent studies seem only to bring rodent levels to within normal limits for human values, and then only when the most severe diets are utilized. This is an observation that should be duly considered when theoretically extrapolating mouse data to human diseases. However, with respect to expressed phenotypes within one model system we can confidently state these to be a function of folate status, regardless of how these values compare to folate levels in independent model systems. One last point to be made is that fortification of grain products is not analogous to the folate supplementation regimes used in these animal studies. Fortification of grain products with folic acid is intended to prevent deficiency of folate, and this public health action has effectively increased average folate levels from 12 ng/mL to approximately 19 ng/mL [2]. This increase is still far less than the 60 ng/mL (rat) and 80 ng/mL (mouse) levels attained on the maintenance (2 mg/kg) diets, such that fears of detrimental effects of folate fortification are unwarranted. That is, folate fortification is not folate supplementation and concerns that fortification of grains with folic acid is dangerous are unsupported by data in the literature.

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Research Article

Sensitization of Cervical Cancer Cells to Cisplatin by Genistein: The Role of NFκB and Akt/mTOR Signaling Pathways

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Cervical cancer is among the top causes of death from cancer in women. Cisplatin-based chemotherapy has been shown to improve survival; however, cisplatin treatment is associated with toxicity to healthy cells. Genistein has been used as an adjunct to chemotherapy to enhance the activity of chemotherapeutic agents without causing increased toxicity. The present study was designed to investigate the effect of genistein (25 μM) on antitumor activity of cisplatin (250 nM) on HeLa cervical cancer cells. We have examined the alterations in expression of NF-κB, p-mTOR, p-p70S6K1, p-4E-BP1, and p-Akt protein levels in response to treatment. The combination of 25 μM genistein with 250 nM cisplatin resulted in significantly greater growth inhibition ($P < 0.01$). Genistein enhanced the antitumor activity of cisplatin and reduced the expression of NF-κB, p-mTOR, p-p70S6K1, p-4E-BP1, and p-Akt. The results in the present study suggest that genistein could enhance the activity of cisplatin via inhibition of NF-κB and Akt/mTOR pathways. Genistein is a promising nontoxic nutritional agent that may enhance treatment outcome in cervical cancer patients when given concomitantly with cisplatin. Clinical trials of genistein and cisplatin combination are warranted to test this hypothesis.

1. Introduction

As of 2008, cervical cancer is the third most common cause of cancer and the fourth most frequent cause of deaths from cancer in women and more than 500,000 new cervical cancer cases and 275,000 deaths were reported worldwide [1]. Although the high incidence rate is disappointing, survival rates of these patients continue to improve with the recent developments in the treatment of this particular cancer type [1, 2]. As the number of studies investigating the application of chemotherapeutical agents as a concomitant treatment method increases, chemoradiotherapy including cisplatin is becoming the recommended method instead of radiotherapy alone [2].

Cisplatin (cis-diamminedichloroplatinum II, CDDP), is an effective agent in the treatment of cervical cancer [3]. However, its usage is limited by its toxicity and acquired chemoresistance throughout the course of treatment [4–6]. To this end, targeted therapies that can differentiate between tumor cells and healthy cells are being developed. A naturally occurring soybean isoflavone, genistein, could inhibit tumor growth and induce apoptosis of tumor cells without damaging the normal cells [7–9].

Genistein (4′,5,7-trihydroxyisoflavone) has a heterocyclic diphenolic structure that is similar to estrogen, but it has a more potent biological activity [10, 11]. Genistein can inhibit tyrosine kinase and inhibit cancer cell proliferation *in vivo* and *in vitro* without causing toxicity to healthy cells [12]. Studies suggest that genistein can also regulate several signaling pathways in cancer cells and promote cancer cell death. Inhibition of Nuclear Factor-kappa B (NF-κB) and attenuation of Akt pathways by genistein have been shown in various cancer types [13–16]. NF-κB not only controls the expression of genes involved in survival and proliferation,

but also plays a key role in apoptosis [17]. Moreover, NF-κB inhibition in tumor cells may result in increased activity of topoisomerase II inhibitors and, hence, this inhibition can be used in anticancer therapy [18].

Phosphatidylinositol 3-kinase (PI3K)/Akt pathway is one of the major growth-factor-induced pathways in tumorigenesis and malignant transformation [19, 20]. Akt pathway activates many downstream signaling pathways responsible for both cell survival and apoptosis [21]. Mammalian target of rapamycin (mTOR) is one of the downstream serine/threonine kinases of PI3K/Akt pathway and regulates cell growth and survival and, thus, it is considered as a valid target for anticancer treatments [22]. mTOR can be either directly phosphorylation-activated by Akt or indirectly activated by Akt through the inhibition of tuberous sclerosis complex 1 and 2 (TSC1/2) and activation of Ras homologue-enriched in brain (Rheb) [23]. mTOR exists as TORC1 and TORC2 complexes. In TORC1 complex, it initiates translation by eukaryotic translation initiation factor (eIF4E) binding proteins (4EBP1) and by ribosomal p70S6 kinase (p70S6K). When mTOR protein phosphorylates 4E-BP1, it dissociates from eIF4E. Once eIF4E is freed from 4e-BP1, it can form complex structures with several other proteins, including eIF4G or eIF4F. When mTOR phosphorylates p70S6K, this kinase phosphorylates S6 ribosomal protein in return [24, 25]. S6 kinase can catalyze phosphorylation and inhibition of insulin receptor substrate (IRS) proteins; then IRS proteins can no longer activate PI3K pathway and this results in an indirect inhibitory effect on Akt [26, 27]. mTOR can also phosphorylate Akt through a possible positive feedback mechanism [28].

In this study, we hypothesized that cisplatin treatment administered together with genistein could potentiate cervical cancer growth inhibition *in vitro* through downregulation of mTOR pathway. To test our hypothesis, we evaluated the effects of genistein and cisplatin on cell growth and apoptosis-related gene expression in HeLa human cervical cancer cell line.

2. Materials and Methods

2.1. Cell Culture and Reagents. The human cervical cancer cell line, HeLa cells (American Type Culture Collection, Manassas, VA) was maintained in RPMI-1640 medium containing 10% heat inactivated fetal bovine serum, 1% L-glutamine, 100 U/mL penicillin G, and 100 μg/mL streptomycin. Cells were incubated in a humidified, 5% CO_2 atmosphere at 37°C. No growth factors were added to the cell culture medium at any time. Genistein (Sigma Chemical Co., St. Louis, MO, USA) was dissolved in 0.1 M Na_2CO_3 to make a 10-mM stock solution. Cisplatin (Sigma Chemical Company, St. Louis, MO) was dissolved in phosphate buffered saline (PBS) to make a 0.5 mM stock solution.

2.2. Cell Viability Assay. Cell viability was determined by MTS Assays. HeLa cells were seeded 3000 cells in a 96-well plate and incubated overnight. Cells ($2–5 \times 10^4$) were treated with genistein (25 μM), cisplatin (250 nM), and their combination treatment for 24 hours. After 24 hours of total treatment, the cells were incubated at 37°C with 1 mg/mL MTT reagent (Sigma, St. Louis, MO) for 2 hours. The formazan crystals were dissolved in isopropanol. Spectrophotometric absorbance of the samples was determined by the ULTRA Multifunctional Microplate Reader (ELx800-BIO-TEK) at 490 nm.

2.3. Western Blot Analysis. HeLa cells were treated with genistein (25 μM), cisplatin (250 nM), and the combination treatment for 24 hours. The total proteins from these samples were extracted. These total proteins were resolved through sodium dodecyl sulfate polyacrylamide gels and then were transferred to a nitrocellulose membrane. After blocking with 5% nonfat dry milk, the membrane was incubated with anti-NF-κB p65, anti-mTOR, anti-70S6K1, anti-4E-BP1, and anti-Akt (Abcam, Cambridge, UK). Primary antibody was diluted (1 : 1000) in the same buffer containing 0.05% Tween-20. The nitrocellulose membrane was incubated overnight at 4°C with protein antibody. The blots were washed and incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (Abcam, Cambridge, UK). Specific binding was detected using diaminobenzidine and H_2O_2 as substrates. Protein loading was controlled using a monoclonal mouse antibody against β-actin antibody (A5316; Sigma). Blots were performed at least three times to confirm data reproducibility. Bands were analyzed densitometrically using an image analysis system (Image J; National Institute of Health, Bethesda, USA).

2.4. Statistical Analysis. To determine the difference in cell viability between experimental sets of cervical cancer cell line, experiments were repeated at least three times and SPSS was used for statistical analysis. Comparisons of treatment outcome were tested for statistical difference by the paired t-test. Statistical significance was assumed at a P value of <0.05.

3. Results

3.1. Genistein Enhances the Inhibitory Effect of Cisplatin on the Proliferation of HeLa Cells. The effects of genistein, cisplatin, and their combination on the proliferation of HeLa cells were evaluated with MTS assay. In MTS assay, cells are treated with a tetrazolium compound, MTS (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide). Since metabolically active cells can reduce MTS to insoluble purple formazan dye products, relative cell viability for each treatment compared to control was measured [29]. Data from this assay showed that combination treatment of genistein and cisplatin enhances the inhibition of cellular growth in HeLa cells.

The rationale for choosing 250 nM cisplatin and 25 μM genistein came from our previous observation that revealed a marked inhibition of cell growth in human cancer cells [7]. HeLa cells were treated with cisplatin and genistein alone and in combination of the two for 24 hours. When compared to controls, combination treatment inhibited the proliferation of HeLa cells to significantly higher extend than either treatment alone. Percent of viable cells after the combination treatment decreased to ~30%, while percents of viable cells for genistein treatment alone and for cisplatin treatment

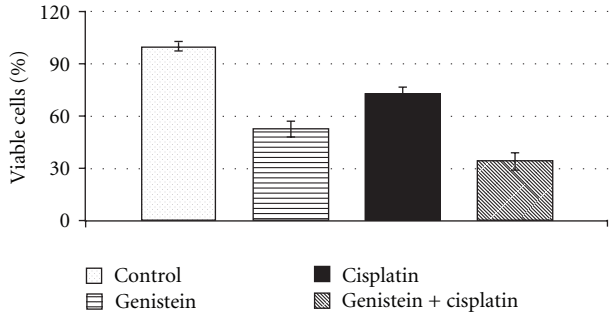

Figure 1: Growth inhibition of human cervical cancer cell lines HeLa treated with genistein, cisplatin, and the combination treatments were evaluated by the MTT assay. HeLa cells were treated with genistein (25 μM), cisplatin (250 nM), and the combination treatment. $^{*}P < 0.05$; $^{**}P < 0.01$.

alone were ~50% and ~70%, respectively (Figure 1). These results suggest that combination of genistein with cisplatin elicited significantly greater growth inhibition in HeLa cells compared to either agent alone. Since we found that genistein could potentiate the inhibition of cancer cell growth, we next tested the expression of possible target proteins which may be involved in the mechanism of genistein and cisplatin.

3.2. Genistein Prevents Cisplatin-Induced Upregulation of NF-κB in HeLa Cells. NF-κB is a transcription factor which plays an important role in apoptosis mechanisms by exerting its regulatory effects on survival genes. Expression level of NF-κB was evaluated at the protein level. By Western Blot analysis, we examined the expression level of NF-κB p65B, a subunit of NF-κB transcription complex, in genistein alone, cisplatin alone, and genistein-plus-cisplatin-treated HeLa cells. Cisplatin alone increased the expression level of NF-κB p65B up to 150%, compared to control, whereas genistein downregulated this subunit to ~75%. When genistein is added to cisplatin treatment, expression level of NF-κB p65B protein is reduced to ~80%. These results suggest that genistein can downregulate the increased expression level of NF-κB induced by cisplatin in HeLa cells (Figure 2(a)).

3.3. Genistein Inhibits Cisplatin-Induced Activation of mTOR Pathway in HeLa Cells. In order to evaluate the involvement of mTOR molecular pathway in the antiproliferative effect of cisplatin and genistein, we assessed the expression levels of p-mTOR, p-p70S6K1, p-4E-BP1, and p-Akt in HeLa cells treated with genistein and/or cisplatin. Genistein reduced the level of phosphorylated mTOR, p70S6K1, 4e-BP1, and Akt induced by cisplatin in HeLa cells (Figures 2(b), 2(c), 2(d), and 2(e)). mTOR is known to regulate initiation of translation through two pathways: S6K and 4E-BP1. As a decrease in expression of mTOR would cause a decrease in expression of these two molecules, this hypothesis is supported by our data.

4. Discussion

There is a need to develop more efficient treatment strategies to increase the efficacy of existing therapies while not compromising the normal cells. Cisplatin is one of the most effective anticancer agents in the treatment of cervical cancer; however, it is associated with severe toxicity and acquired drug resistance after therapy. Severe renal, neurologic, and gastrointestinal side effects and acquired chemoresistance are the major reasons of cisplatin treatment failure. To overcome the limitations of cisplatin treatment, combination with targeted therapy using naturally occurring compounds was suggested [30–32]. Many natural compounds with known anticancer activity have been used including sulforaphane [33, 34] and genistein [35]. In the light of these previous studies we chose genistein as a nontoxic nutritional agent to augment the efficacy of cisplatin treatment in HeLa cells.

In this study, we observed the superiority of genistein plus cisplatin combination compared to cisplatin alone in inhibition of the growth of HeLa cervical cancer cells *in vitro*. The effect of genistein on cisplatin's anticancer activity has been previously reported for ovarian cancer [36] and pancreatic cancer [35]. We investigated the therapeutic effect of genistein and cisplatin in the HeLa cells and found a statistically significant inhibition of cell growth when cells were treated with a combination of genistein and cisplatin, compared to either agent alone. Growth inhibition of HeLa by cisplatin was augmented by genistein, thereby obviating the need to further increase the concentration of cisplatin. The effect of genistein-mediated enhanced efficacy of cisplatin in cervical cancer cells was demonstrated for the first time in this study.

Activation of mTOR signaling pathway is associated with cell survival in cervical cancer cells [37]. We have found increased mTOR expression after cisplatin treatment which could be prevented by the addition of genistein, a mechanism first shown in the present study. The combination of cisplatin and genistein could be a promising strategy in the treatment of cervical cancer. mTOR activity can be monitored by phosphorylation of S6K, 4E-PB1 proteins [38]; we also observed increased expression of these proteins with cisplatin treatment which could be abrogated by genistein. When HeLa cells were treated with cisplatin alone, the expression levels of phosphorylated mTOR, p70S6K1, and 4E-BP1 increased up to 140%, 170%, and 150%, respectively. However, addition of genistein downregulated these cisplatin-induced proteins by 70%. These results suggest that cisplatin upregulates mTOR pathway and genistein prevents this upregulation by downregulating phosphorylated p70S6K1 and 4E-BP1 proteins. We also observed that this activity is associated with downregulation of phosphorylated Akt, which suggests that decrease in the expression of mTOR pathway is probably mediated via Akt or a decrease in this pathway negatively regulates and inactivates Akt.

In this study, we also investigated the effects of genistein and cisplatin on NF-κB, which is known to be upregulated upon cisplatin treatment [36]. Similar to previous observations, genistein decreased the expression of cisplatin-induced NF-κB in HeLa cells. These results suggest a molecular mechanism involving both NF-κB and mTOR pathways induced by cisplatin and inhibited by genistein.

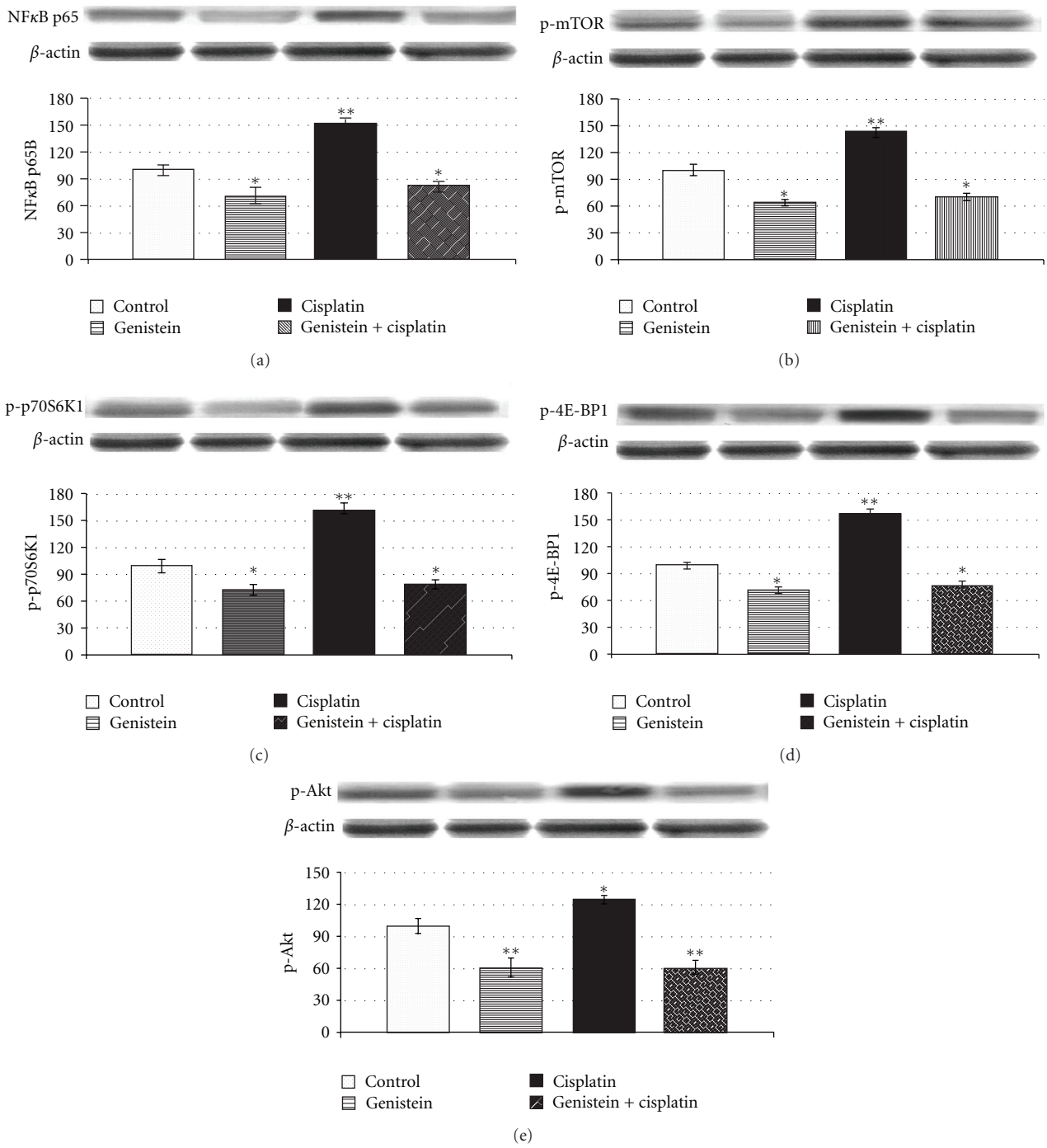

FIGURE 2: The intensity of the bands was quantified by the densitometric analysis. The expression of (a) NF-κB, (b) p-mTOR, (c) p-p70S6K1, (d) p-4E-BP1, and (e) p-Akt in HeLa cells. Cells untreated or treated with 25 μM genistein, 250 nM cisplatin (Cis), and the combination (genistein + cisplatin). β-actin antibodies were used as internal controls for equal loading of proteins. Data are percent of the control. $^{*}P < 0.05$; $^{**}P < 0.01$.

5. Conclusion

In conclusion, cisplatin treatment is potentiated with genistein in HeLa cells by regulating NF-κB, Akt, and mTOR pathways which are critical for cell survival and apoptosis. Our findings suggest that cisplatin and genistein combination could be used to improve the treatment outcome in cervical cancer. This combination is a less toxic option in the treatment of cervical cancer, especially in the presence of chemoresistance to cisplatin. Future clinical trials are warranted to investigate the combination of cisplatin and genistein in patients with cervical cancer.

Conflict of Interests

The authors have declared no conflict of interests.

Acknowledgment

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Review Article

DNA Repair and Cancer Therapy: Targeting APE1/Ref-1 Using Dietary Agents

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Epidemiological studies have demonstrated the cancer protective effects of dietary agents and other natural compounds isolated from fruits, soybeans, and vegetables on neoplasia. Studies have also revealed the potential for these natural products to be combined with chemotherapy or radiotherapy for the more effective treatment of cancer. In this paper we discuss the potential for targeting the DNA base excision repair enzyme APE1/Ref-1 using dietary agents such as soy isoflavones, resveratrol, curcumin, and the vitamins ascorbate and α-tocopherol. We also discuss the potential role of soy isoflavones in sensitizing cancer cells to the effects of radiotherapy. A comprehensive review of the dual nature of APE1/Ref-1 in DNA repair and redox activation of cellular transcription factors, NF-κB and HIF-1α, is also discussed. Further research efforts dedicated to delineating the role of APE1/Ref-1 DNA repair versus redox activity in sensitizing cancer cells to conventional treatment are warranted.

1. Introduction

Despite the "war on cancer" initiated by the signing of the National Cancer Act of 1971, cancer remains a major public health concern in the United States accounting for approximately 1 in 4 deaths [1]. Current conventional cancer treatment involves the use of chemotherapy or radiotherapy, either alone or in combination. The central mechanism by which chemotherapy or radiation exert their cytotoxic effects is directly related to their ability to cause DNA damage. Limitations exist, however, for these treatment options when used as single modalities for most solid tumors as cancer cells are known to be highly heterogenous and display deregulation of multiple cellular signaling pathways. In order to improve cancer treatment outcomes, new strategies must be investigated. Novel concepts include using targeted therapies and combining drugs with dietary agents for improved cancer cell death and reduced residual toxicity.

Sensitizing cancer cells to DNA damaging agents by targeting DNA repair pathways is an emerging concept that is receiving much deserved attention [2]. Efficient DNA repair is an important mechanism by which cancer cells exert therapeutic resistance. Thus, altering the ability of a cancer cell to respond to DNA damaging agents should render a cell more susceptible to death. This concept is further supported by research demonstrating that polymorphisms in DNA repair are associated with increased risk for cancer, influence the natural history and progression of the disease, and predict response to chemotherapy and radiation [3–6]. It is therefore worth pursuing new strategies of cancer therapy that target DNA repair.

Numerous studies also support the notion that diet could influence cancer development, progression, metastasis and mortality [7]. Furthermore, these studies suggest that susceptibility to various cancers is due to environmental factors, that is, diet, rather than genetic differences.

The potential for herbs and other plant-based formulations to act as antioxidants has also been increasingly recognized in the prevention and treatment of cancers [8–10]. These dietary compounds include, but are not limited to, soy isoflavones, resveratrol, lycopene, thymoquinones and their derivatives, green tea polyphenols, and curcumin. All have been recognized as cancer chemopreventive agents because of their anticarcinogenic activity, yet also exert antitumor activities through regulation of different cell signaling pathways. Therefore, the use of dietary agents to potentiate conventional cancer treatment is a promising area for investigation [11–16].

The purpose of this paper is to understand the use of dietary agents, in particular soy isoflavones, as DNA repair inhibitors. Specifically, we will discuss targeting the DNA base excision repair (BER) enzyme apurinic/apyrimidinic endonuclease 1/redox-factor-1 (APE1/Ref-1), a multifunctional protein involved in both DNA repair and redox signaling, whose expression is altered in numerous cancers including prostate, colon, ovarian, cervical, and germ cell tumors [17]. Elevated levels of APE1/Ref-1 have been linked to resistance to chemotherapy, poor prognosis, and poor survival [18–20]. Selective targeting of this DNA repair enzyme using RNA interference, antisense oligonucleotides, and dietary agents has been shown to be effective in sensitizing cancer cells to both radiation and chemotherapy *in vivo* and *in vitro* [11, 12, 18–20]. Furthermore, the use of specific small-molecule inhibitors blocking either APE1/Ref-1 repair or redox functions, but not both, is currently under investigation [21]. However, research utilizing dietary approaches that inhibit DNA repair enzymes is relatively scarce and the need for further studies will be emphasized.

2. APE1/Ref-1: An Overview

Apurinic/apyrimidinic (AP) endonuclease 1 (APE1) is a multifunctional protein involved in the maintenance of genomic integrity and in the regulation of gene expression. After initial discovery in *E. coli* [22], APE1 was purified from calf thymus DNA and characterized as an endonuclease that cleaves the backbone of double-stranded DNA containing AP sites [23, 24]. APE1 homologues were subsequently identified and characterized in yeast as *APN1* [25], mice as *Apex* [26, 27], and humans as *HAP1* [28]. In addition to its major 5′-endonuclease activity, APE1 expresses minor 3′-phosphodiesterase, 3′-phosphatase, and 3′ → 5′ exonuclease activities [29]. APE1 is the primary enzyme responsible for recognition and incision of noncoding AP sites in DNA resulting from spontaneous, chemical, or DNA glycosylase-mediated hydrolysis of the *N*-glycosyl bond initiated by the DNA base excision repair (BER) pathway. AP sites are particularly common, arising at the rate of approximately 50,000–200,000 per cell per day under normal physiological conditions [30, 31]. If unrepaired, these sites promote cell death by serving as blocks to DNA replication [32], stalling RNA transcription [33], or promoting double-strand DNA breaks [34], thus, highlighting the potential of APE1 to serve as a target for cancer therapeutics.

BER is the main pathway responsible for repairing AP sites in DNA and is initiated by DNA damage recognition enzymes, that is, monofunctional or bifunctional DNA glycosylases, in addition to APE1-mediated AP site recognition (Figure 1). In monofunctional glycosylase-initiated BER (MFG-BER), a damaged or improper base is recognized and removed by enzymatic hydrolysis of the *N*-glycosyl bond resulting in the formation of an AP site. This serves as a substrate for APE1 which then incises the DNA backbone immediately 5′ to the AP site via its 5′-endonuclease activity, producing a single-strand break with a normal 3′-hydroxyl group and an abnormal 5′-deoxyribose-5-phosphate (dRP) residue [35]. DNA polymerase β (β-pol) then inserts a new base followed by the coupled excision of the abnormal 5′-dRP (Figure 1) [36]. In bifunctional glycosylase-initiated BER (BFG-BER), a damage-specific DNA glycosylase recognizes and removes the damaged base followed by incision of the DNA backbone by the associated AP lyase activity, yielding a normal 5′-terminal deoxynucleoside-5′-phosphate residue and an abnormal 3′-terminal α,β-unsaturated aldehyde residue that must be processed by APE1 3′-phosphodiesterase activity prior to repair completion (Figure 1) [35, 37]. BER may then proceed by one of two pathways: (i) short patch BER, a β-pol-mediated single nucleotide insertion, similar to MFG-BER, or (ii) long patch BER, that is, multiple nucleotide strand-displacement synthesis which is required to process modified (i.e., reduced, oxidized) AP sites and involves components of the DNA replication machinery [38]. Repair is completed upon the nick-sealing activity of DNA ligase complexes (Figure 1) [39].

Independent of its discovery as a DNA repair protein, APE1 was also characterized as Ref-1, for redox effector factor-1, the nuclear factor responsible for reducing the transcription factor AP-1 [40, 41]. Since this initial discovery, APE1/Ref-1 was characterized as a redox activator of a number of additional transcription factors known to be involved in cancer cell signaling, such as NF-κB, HIF-1α, p53, and others (Figure 2) [17]. While the exact mechanism of the redox change has yet to be elucidated, it is known that oxidation of a cysteine residue abolishes DNA binding, whereas reduction to a sulfhydryl state promotes DNA binding [42]. The redox state of cysteine residues also influences the various properties of proteins, including protein stability, structure, and enzymatic activity [42]. The discovery of APE1/Ref-1 as a regulator of transcriptional activity could underscore the importance of its involvement in an array of physiological functions including cellular growth and differentiation, cell cycle control, apoptosis, and angiogenesis. All of which have implications for the development of cancer therapeutics.

3. Phenotypic Effect of APE1/Ref-1

The importance of APE1/Ref-1 in normal cellular function is highlighted by research demonstrating the embryonic lethality of mice with homozygous deletion of the APE1 gene ($Apex^{-/-}$), but heterozygous mice survive and are

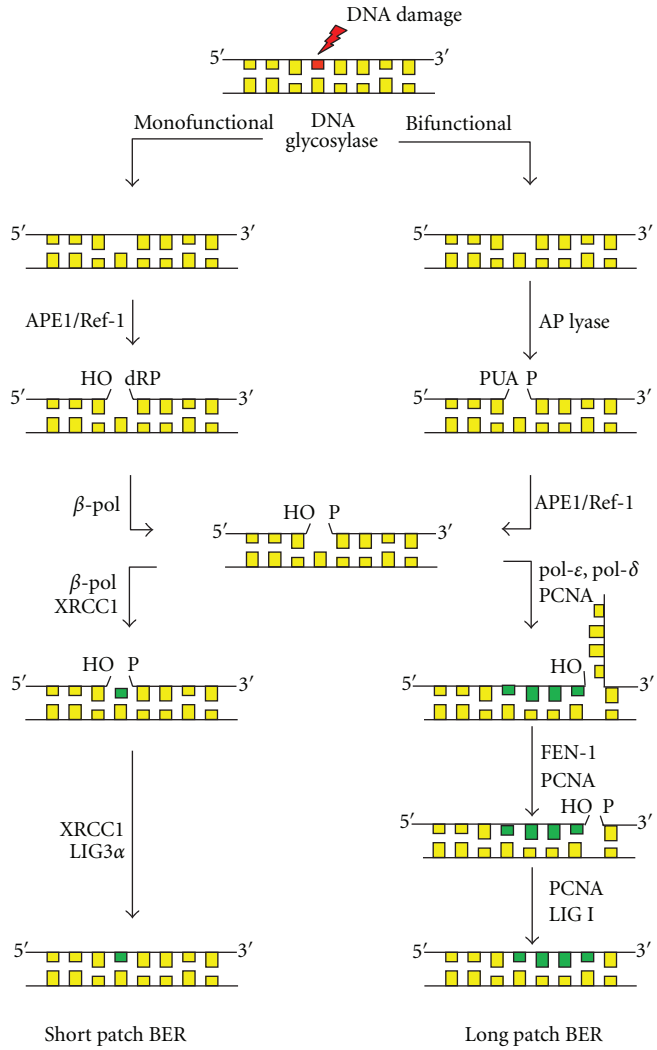

Figure 1: The DNA base excision repair (BER) pathway. DNA glycosylases initiate BER by recognizing and removing DNA damage forming an apurinic/apyrimidinic (AP) site. In MFG-BER, APE1/Ref-1 hydrolyzes the phosphate bond 5′ to the AP site leaving a 3′-OH group and a 5′-deoxyribose phosphate (5′-dRP) termini. DNA polymerase β (β-pol) then excises the 5′-dRP moiety generating a 5′-phosphate (5′-P). If the pathway is initiated by a bifunctional DNA glycosylase, removal of the damaged base and AP site formation is followed by AP lyase activity that hydrolyzes the 3′-bond to the AP site, resulting in a phospho-α,β-unsaturated aldehyde AP site (PUA). APE1/Ref-1 processes this site resulting in a 3′-OH group. BER then proceeds via short-patch or long-patch BER. In short-patch BER, β-pol inserts a single nucleotide in the AP site and LigIIIα ligates the DNA backbone. In long-patch BER, pol δ/ε inserts 2-8 nucleotides in the AP site. The resulting DNA flap is excised by the FEN1/PCNA endonuclease complex and the DNA backbone ligated by Ligase I (LIG1).

fertile ($Apex^{+/-}$) [43–45]. Cell lines completely deficient for APE1/Ref-1 are also nonviable and further demonstrate its importance in cell survival and propagation [45]. Pursuing APE1/Ref-1 inhibition as a strategy for cancer cell therapy is justified based on the following observations. (1) APE1/Ref-1 expression and/or activity are upregulated or dysregulated in many types of cancer, including prostate, ovarian, cervical, pancreatic, colon, germ cell tumors, and rhabdomyosarcomas [17–20, 46–48]. (2) Reduction in APE1/Ref-1 using RNA interference or antisense technology *in vitro* or in studies with $Apex^{+/-}$ mice potentiates the cytotoxicity of many laboratory and clinical DNA damaging agents including methylmethane sulfonate (MMS), H_2O_2, 2-nitropropane (2-NP), bleomycin, temozolomide (TMZ), melphalan, cisplatin, gemcitabine, and radiation [17–20, 46–48]. (3) Elevated expression of APE1/Ref-1 is associated with increased resistance to radiation and chemotherapy, incomplete treatment response, poor survival and prognosis, and increased level of angiogenesis [17–20, 46–48].

We have extensively characterized a mouse containing a heterozygous gene-targeted deletion of the APE1/Ref-1 gene ($Apex^{+/-}$) [48, 49]. Our studies demonstrated that APE1/Ref-1 haploinsufficient ($Apex^{+/-}$) mice show tissue-specific differences in BER capacity as characterized by an *in vitro* G : U mismatch repair assay. Others have shown that these mice display increased spontaneous mutagenesis

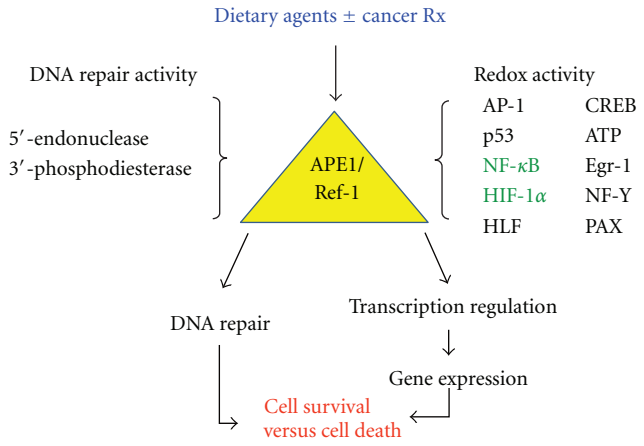

Figure 2: The dual functions of APE1/Ref-1. As a DNA repair protein, APE1/Ref-1 functions as the primary enzyme responsible for recognition and repair of mutagenic apurinic/apyrimidinic (AP) sites in DNA as part of the base excision repair (BER) pathway. As a redox protein, APE1/Ref-1 functions as an activator of transcription factors involved in multiple cellular processes, including AP-1, p53, NF-κB, HIF-1α, and others. Activation involves the reduction of a cysteine residue to a sulfhydryl state. Dietary agents may target APE1/Ref-1 DNA repair or redox activities or both, causing multiple downstream effects.

in liver and spleen [50]. Furthermore, embryonic fibroblasts and brain cells obtained from Apex$^{+/-}$ mice are more susceptible to oxidative stress [51].

Previous studies have indicated that downregulation of APE1/Ref-1 may promote a DNA damage-hypersensitive phenotype [51]. To determine the functional importance of decreased APE1/Ref-1 in haploinsufficient mice, we analyzed the effect of reduced APE1/Ref-1 on 2-Nitropropane- (2-NP-) induced oxidative DNA damage *in vivo* [47, 48]. 2-NP is a known hepatocarcinogen and inducer of oxidative DNA damage in the form of increased 8-hydroxydeoxyguanosine, DNA single-strand breaks, p53 levels, and β-pol expression and BER activity *in vivo* [47]. Previously, we have measured the presence of AP sites, single-strand breaks, and aldehydic lesions in isolated liver DNA from APE1/Ref-1 haploinsufficient mice and observed no significant difference in DNA damage accumulation as a result of reduced APE1/Ref-1 [49]. The lack of damage accumulation in untreated Apex$^{+/-}$ mice suggested that APE1/Ref-1 haploinsufficiency in liver does not cause an accumulation of genotoxic DNA repair intermediate products under baseline conditions. In line with previous studies from our laboratory [47], we have demonstrated a significant increase in 3′-OH-containing single-strand breaks in response to oxidative stress. However the level of detectable single strand breaks (SSB's) in the liver tissue of 2-NP-treated Apex$^{+/-}$ mice was found to be significantly lower than its wildtype counterpart while the level of aldehydic lesion was significantly higher. We suggest that the processing of oxidized bases by a bifunctional DNA glycosylase such as OGG1 (8-oxoguanine DNA glycosylase) could result in generation of aldehydic blocking lesions at 3′ end. Inability to process these 3′ blocking groups in the absence of the 3′-phosphodiesterase activity of Apex in Apex$^{+/-}$ mice [37], could result in lower detection of endonuclease-mediated single-strand breaks in the heterozygous animal.

Reports to date have shown that APE1/Ref-1 is inducible in response to various forms of oxidative stress [49, 51–58]; however, it is currently unclear whether this response is due to APE1/Ref-1 repair activity versus redox regulatory activity, or both. Our studies in Apex$^{+/-}$ mice indicate that APE1/Ref-1 is indeed an inducible protein, with concomitant changes in NF-κB, emphasizing its role as a redox protein [48]. We have confirmed that APE1/Ref-1 is indeed an inducible protein [48]. While fold increase is the same in response to oxidative stress across the genotypes, the total accumulative level of APE1/Ref-1 protein is lower in the liver of Apex$^{+/-}$ mice; that is, even though the intact allele is induced in response to 2-NP, it does not compensate for the lost allele. In line with these findings, the Apex$^{+/+}$ mice showed a significant increase in APE1/Ref-1 redox activation of NF-κB when exposed to 2-NP. Thus, the ultimate level of NF-κB activation in response to oxidative stress was significantly attenuated in the heterozygous (Apex$^{+/-}$) animals.

It is well established that NF-κB is a mediator of inflammatory responses, promoting cell proliferation and survival by inhibiting cell cycle arrest and apoptosis. Thus, reduced activation of NF-κB (and possibly other APE1/Ref-1 redox-dependent transcription factors) in response to oxidative stress in Apex$^{+/-}$ mice may prove detrimental owing to alterations in the signaling pathways necessary to differentiate between DNA repair and cell survival versus apoptosis. However, although the fold increase in response to oxidative stress is the same compared to wild-type (Apex$^{+/+}$) mice, the total cumulative level of APE1/Ref-1 protein is lower. Hence, the intact allele in wild-type mice does not compensate for the lost allele in Apex$^{+/-}$ mice. Thus, reduced activation of NF-κB (and possibly other APE1/Ref-1 redox-dependent transcription factors) in response to oxidative stress in Apex$^{+/-}$ mice may prove detrimental owing to alterations in the signaling pathways necessary to differentiate between DNA repair and cell survival versus apoptosis.

When examining the effect of reduced APE1/Ref-1 on DNA damage accumulation, Apex$^{+/-}$ mice expressed a BER phenotype that is more susceptible to accumulation of DNA damage in response to oxidative stress as a result of reduced APE1/Ref-1 3′-phosphodiesterase activity [48]. Reduced APE1/Ref-1 in haploinsufficient mice also resulted in a differential impact on BER, depending upon the initiating glycosylase [48]. Oxidative stress resulted in increased MFG-BER initiated by uracil DNA glycosylase (UDG), but a significant decline in the repair or oxidized bases (8-OHdG) initiated by OGG1 (8-oxoguanine DNA glycosylase) in BFG-BER (Figure 1) [48]. The failed upregulation of BFG-BER and accumulation of repair intermediates in Apex$^{+/-}$ mice exposed to oxidative stress coincided with increased cell death as indicated by increased expression of apoptotic markers such as GADD45g, p53, and caspase-3 activity [48].

Taken together, these results indicate that when APE1/Ref-1 is compromised, cells become more susceptible

to oxidative stress primarily as a result of reduced APE1/Ref-1 redox activity and 3′-phosphodiesterase repair activity, thus impacting cell survival and pushing cells towards apoptosis. These findings have great clinical relevance for cancer as development of therapeutics targeting APE1/Ref-1 DNA repair or redox activities, or both, could potentiate current cancer cell treatment strategies.

Interestingly, dietary agents such as soy isoflavones have been shown to interfere with APE1/Ref-1 repair and redox activity resulting in potentiation of radiotherapy for cancer cells [11, 12, 59, 60].

4. Dietary Modulation of APE1/Ref-1

Epidemiological studies indicate an inverse association between cancer risk and consumption of a diet rich in fruits and vegetables [7]. The cancer-inhibitory potential of nutrient and nonnutrient components, that is, phytochemicals, from plants has been confirmed in animal models [7]. Dietary sources of phytochemicals include whole grain cereal foods, seeds, soybean products (mainly isoflavones), berries or grapes (resveratrol), and nuts (mainly lignans). Emphasizing a healthy diet is relevant for cancer prevention. In addition, the use of diet as a safe and healthy supplement to conventional cancer therapy has also gained significant interest in the scientific community. Nontoxic "natural products" found in the diet have been shown to be effective in combination with conventional agents for the treatment of cancer [11–16] and such strategies are worth exploring. Here, we will review studies using dietary agents (soy isoflavones, curcumin, and resveratrol), including antioxidants (selenium, ascorbate, and α-tocopherol), and discuss their effects on APE1/Ref-1.

4.1. Soy Isoflavones. Soy isoflavones, which include genistein, daidzein, and glycitein, are plant estrogens with potent anti-oxidant and anti-inflammatory properties. An inverse association between consumption of soy isoflavones and cancer incidence has been widely documented [11–16]. We and others have shown that soy isoflavones enhance the efficacy of chemotherapy and radiation therapy of multiple cancers models *in vitro* and *in vivo* [59, 60] and in early clinical trials [61]. In addition to their use as potent adjuvant therapies, soy isoflavones could also potentially protect normal tissues from treatment-induced toxicity [11, 12, 61] and have generated much interest in the clinical research community [16].

Soy isoflavones (or pure genistein) inhibited APE1/Ref-1 expression in prostate cancer cells in a time- and dose-dependent manner [59]. The nuclear expression of APE1/Ref-1 was increased by radiation, probably representing an early event in the cell response to radiation because of its role in BER [59]. Pretreatment of prostate cancer cells with soy isoflavones inhibited both the increased expression and the nuclear localization of APE1/Ref-1 induced by radiation [59]. These data were reproduced in A549 nonsmall-cell lung cancer cells, demonstrating that soy isoflavones caused a decrease in APE1/Ref-1 expression and inhibited upregulation of APE1/Ref-1 expression induced by radiation [60]. It is conceivable that inhibition of APE1/Ref-1 levels by soy isoflavones could render the cancer cells more radiosensitive. Some attempts have been made to correlate APE1/Ref-1 levels of expression with tumor sensitivity to radiation therapy as increased APE1/Ref-1 expression promoted tumor resistance to ionizing radiation [62]. Conversely, decreased APE1/Ref-1 levels in RNAi-treated human osteogenic sarcoma cells led to enhanced cell sensitization to the DNA damaging agents including ionizing radiation [63].

4.1.1. Effect of Soy on APE1/Ref-1 DNA Repair Activity. To investigate further the role of APE1/Ref-1 function in the mechanism of interaction between soy isoflavones and radiation, the formation and repair of DNA double-strand breaks (DSBs) induced by radiation were studied. Ionizing radiation causes rapid phosphorylation of the nucleosomal histone protein H2AX at Ser 139 (γ-H2AX), occurring at sites of DNA DSBs, which can be visualized as fluorescent foci by immunostaining [64, 65]. Formation of γ-H2AX foci occurs within minutes after production of DSBs by ionizing radiation, and the loss of γ-H2AX foci after several hours can be attributed to DNA repair enzymes [64, 65]. In A549 nonsmall cell lung cancer cells, a large number of γ-H2AX foci occurred by 1 h after 3 Gy radiation, but drastically decreased at 24 h after radiation, suggesting that A549 cells activated DNA repair mechanisms. Interestingly, we found that soy isoflavones also cause DSBs [60]. However, in contrast to radiation, the number of γ-H2AX foci increased and persisted over time in soy isoflavones pretreated cells, probably interfering with DNA repair mechanisms [60]. Importantly, the combination of soy isoflavones and radiation caused an increase in frequency and intensity of γ-H2AX foci, which were maintained at 24 h, indicating both increased DNA damage and inhibition of repair [60]. Our novel findings on induction and kinetics of DSBs formation by soy isoflavones suggest that soy isoflavones disrupt DNA repair processes and potentially sensitizes nonsmall cell lung cancer cells to the cytotoxic effect of radiation [60]. Furthermore, these data are also consistent with the inhibition of the radiation-induced upregulation of the DNA repair enzyme APE1/Ref-1 by soy isoflavones in A549 cells, which could contribute to alterations in DNA repair mechanisms [60]. In contrast, cells treated with radiation alone showed a significant increase in APE1/Ref-1 within 5 h after radiation, which could be associated with the loss of γ-H2AX foci.

To determine if the soy-mediated decrease in APE1/Ref-1 expression is involved in the mechanism of soy inhibition of DNA repair, two different APE1/Ref-1 inhibitors, E3330 and methoxyamine, were tested [60]. E3330, a novel quinine derivative shown to inhibit the redox activity of APE1/Ref-1 [17–21], did not alter the repair of radiation-induced DSBs over time. However, methoxyamine, an alkoxyamine derivative and indirect inhibitor of APE1/Ref-1 endonuclease activity [17–21], partially blocked the decrease in radiation-induced DSBs. These data indicate partial mitigation of radiation-induced BER by methoxyamine, akin to the effect of soy when it is combined with radiation. Methoxyamine also increased cell killing mediated by soy isoflavones as well as that by soy combined with radiation, suggesting that

additional DNA repair inhibition of APE1/Ref-1 results in further cell killing [60]. These findings suggest that inhibition of APE1/Ref-1 DNA repair activity by soy isoflavones is involved in the mechanism by which soy isoflavones potentiate radiation-induced cancer cell killing.

4.1.2. Effect of Soy on APE1/Ref-1 Redox Activity. In numerous studies, NF-κB was shown to be an important molecular target of soy isoflavones in cancer cells [13–16]. The inhibition of NF-κB DNA binding activity by soy isoflavones alone or combined with radiation correlated with the downregulation of APE1/Ref-1 expression [59]. Overexpression of APE1/Ref-1, obtained by cDNA transfection of PC-3 cells, caused a concomitant increase in NF-κB DNA binding activity. Moreover, soy isoflavones treatment of APE1/Ref-1 overexpressing PC-3 cells significantly inhibited APE1/Ref-1 expression with a corresponding decrease in the NF-κB DNA binding activity [59]. Thus, in addition to alteration of the DNA repair activity of APE1/Ref-1, soy isoflavones also affected the redox activation function of APE1/Ref-1 (Figure 2). These findings further confirm that soy isoflavones disrupt molecular cross-talks between APE1/Ref-1 and NF-κB which are two critical molecules essential for cell survival pathways.

Another critical signaling pathway upregulated by radiation-induced oxidative stress is the transcription factor hypoxia-inducible factor (HIF-1α), which is induced by hypoxia. HIF-1α is responsible for the activation of more than 60 downstream target genes involved in angiogenesis, tumor growth, and invasion [13–16]. Interestingly, APE1/Ref-1 is also responsible for redox-activation of HIF-1α (Figure 2). In the hypoxic response, cellular levels of HIF-1α and APE1/Ref-1 redox stabilization of the HIF-1α protein are critical for its nuclear translocation and DNA binding and transcriptional activity [61]. Studies on cellular localization of HIF-1α demonstrated that soy isoflavones inhibited nuclear translocation of HIF-1α protein, a process which is upregulated by radiation but suppressed by pretreatment with soy isoflavones [66]. Therefore, APE1/Ref-1 downregulation by soy isoflavones could play a central and pivotal role in radiosensitization of prostate cancer cells by affecting HIF-1α pathway. Radiation induced HIF-1α expression and DNA-binding activity *in vitro* but both were abrogated by pretreatment of PC-3, C4-2B, and A549 cells with soy isoflavones in [60, 66]. Therefore, soy isoflavone-mediated inhibition of HIF-1α activation by oxidative stress could render cancer cells more radiosensitive.

These findings demonstrate the molecular cross-talk between APE1/Ref-1, NF-κB, and HIF-1α and indicate a critical role for APE1/Ref-1 in the mechanism of interaction between soy isoflavones and radiation that results in the inhibition of NF-κB, and HIF-1α transcription of genes essential for tumor cell survival, tumor growth, and angiogenesis (Figure 2). Our studies confirm that soy isoflavones exert pleiotropic molecular effects in cancer cells, which result in the regulation of multiple signal transduction pathways involved in tumor cell growth and proliferation.

4.2. Resveratrol. Resveratrol (3,5,4′-trihydroxy-*trans*-stilbene), is a naturally occurring polyhydroxylated stilbene that is widely present in grapes, red wine, mulberries, and other edible plants. Resveratrol prevented the development of carcinogen-induced skin cancer in mice and was effective in all stages of carcinogenesis [67]. Resveratrol has also been shown to be effective in the prevention of DMBA-induced mammary carcinogenesis [68]. Resveratrol induced prostate cancer cell apoptosis in multiple cell lines and suppressed the progression of prostate cancer in TRAMP mice [69–71]. Resveratrol was also effective against tumors of the liver, pancreas, gastrointestinal tract, lung, and soft tissues [72–76]. It has also been shown to enhance the therapeutic effects of 5-FU in a murine model of liver cancer [77]. Phase I studies with resveratrol have been promising and demonstrated the clinical safety of oral resveratrol up to 5 g per day [78].

Although studies have shown that resveratrol exerts protective effects against experimentally induced carcinogenesis, the molecular mechanism(s) by which this occurs is largely unknown. It is believed to act as an antioxidant. In studies using human melanoma cells, resveratrol was shown to inhibit, in a dose-dependent manner, the APE1/Ref-1-mediated DNA-binding of AP-1. Resveratrol was also shown to inhibit APE1/Ref-1 endonuclease activity and render melanoma cells more sensitive to treatment with the alkylating agent dacarbazine [79]. These findings suggest a role for APE1/Ref-1 redox and repair activity in the mechanism of action of resveratrol and the need for expanding further on these studies.

4.3. Curcumin. Curcumin, isolated from the plant root of *Curcuma longa*, is the major yellow pigment in turmeric, a widely used spice, and well-known medicinal agent in Southeast Asia. Curcumin has been shown to exhibit antitumor effects in multiple cancer cell lines and animal models [80] and to enhance the efficacy of chemotherapeutic drugs such as 5-FU, gemcitabine, and the vinka alkaloid vinorelbine [81–83]. Curcumin also has synergistic activity with other dietary agents such as genistein and green tea [84, 85]. A phase I clinical trial showed that curcumin is safe up to 8 grams per day [86].

The molecular mechanism of action of curcumin has been shown to involve the interruption of cancer initiation or suppression of tumor promotion and progression [87, 88]. Several studies have demonstrated the inhibitory effects of curcumin on colon carcinogenesis [89, 90], chemically-induced skin cancer [91], and DMBA-induced oral cancer [92]. Curcumin also inhibits the growth of different types of cancer cells *in vitro* and in xenograft models by inducing cell cycle arrest and apoptosis [93, 94]. Interestingly, a recent study evaluating the use of curcumin in protecting and treating carbon tetrachloride-induced liver fibrogenesis in rats showed that APE1/Ref-1 levels correlated with reduced markers of liver damage [95]. The mechanism of both liver fibrogenesis and carcinogenesis involves the cellular response to oxidative stress. Therefore, further study examining the

effects of curcumin on APE1/Ref-1 expression and activity in cancer cells lines or tumor models is warranted, as well as to determine a synergistic effect with traditional antitumor agents.

4.4. Antioxidants

4.4.1. Selenium. Selenium is found in plentiful amounts in dairy, eggs, fish, meat, grains, and nuts. Selenium in the form of selenocysteine is a major constituent of many antioxidants known as selenoproteins. The cancer preventive effect of selenium is believed to occur by reducing the formation of oxidative DNA damage and increasing DNA repair. The active species of selenium include its methylated metabolite selenomethionine (SeMet).

Selenium in the form of SeMet was reported to promote BER activity by p53 activation in normal human fibroblasts *in vitro* [96]. Selenium-induced p53 activation promotes BER activity by reducing specific cysteine residues in p53. A dominant-negative APE1/Ref-1 redox mutant blocks reductive activation of p53 by selenium. Selenium was also shown to stimulate the activity of thioredoxin reductase (TRX), a selenoprotein [97]. These data suggest that selenium reduces p53 through interactions involving TR, which reduces TRX and APE1/Ref-1, as well as redox interactions between APE1/Ref-1 and p53. Selenium has also been show to inhibit DNA binding of transcription factors AP-1, NF-κB, and the BER DNA glycosylase FPG [97]. These data imply that selenium may reduce cancer incidence through modulation of DNA repair, cellular redox status, and transcriptional responses to oxidative stress. It also suggests that the redox function of APE1/Ref-1 is a major component of this interaction.

4.4.2. Ascorbate and Alpha-Tocopherol. DNA damage is a well known mechanism of carcinogenesis and both endogenous and exogenous sources of DNA damage, including oxidative DNA damage, have been extensively characterized [98]. Many natural compounds found in the diet exert antioxidant effects and have been under extensive investigation for their cancer chemopreventive potential [99]. These agents are believed to act by reducing the oxidative burden in cells as well as to promote increased DNA repair. APE1/Ref-1 is a key enzyme in the repair of oxidative DNA damage and studies have shown that mice heterozygous for the APE1/Ref-1 gene are abnormally sensitive to increased oxidative stress and exhibit increased biomarkers of oxidative stress and reduced survival [100].

Anti-oxidants such as ascorbate (vitamin C) and α-tocopherol (vitamin E) are found in citrus fruits, broccoli, and tomatoes and have been reported to initiate physiological responses that lower cancer risk by scavenging free radicals or reacting with their byproducts [99]. Supplementation of APE1/Ref-1 heterozygous mice with ascorbate and α-tocopherol restored the biomarkers of oxidative stress to normal and improved longevity in these mice [43]. These results were consistent with the hypothesis that humans with an APE1/Ref-1 deficiency are more susceptible to cancer through promotion of a DNA damage phenotype and that a diet rich in fruits and vegetables is protective, when DNA repair is compromised.

5. Conclusion and Future Directions

The use of nutrition intervention as an adjuvant to conventional cancer therapy has great therapeutic potential. Several studies have been referenced demonstrating the effect of natural dietary agents on the inhibition of cancer cell growth and their role in the prevention of neoplasia. These effects involve the modulation of multiple DNA repair genes, including but not limited to APE1/Ref-1, and genes involved in cell cycle progression, apoptosis, and the regulation of tumor cell invasion and metastasis [2].

In this paper, we discussed the use of dietary agents in targeting APE1/Ref-1 in order to enhance cancer therapy and prevention. APE1/Ref-1 is constitutively activated in cancer cells and upregulated further in response to certain chemotherapeutics and radiation damage, but is inhibited by dietary agents, such as soy isoflavones, leading to increased cell killing and tumor growth inhibition. We suggest that APE1/Ref-1, a protein involved in both DNA repair and redox activation of transcription factors such as NF-κB and HIF-1α, could play a critical role in the mechanism of interaction between dietary agents and radiation or chemotherapeutic agents. Dietary targeting of APE1/Ref-1 inhibited radiation-induced activation of both its DNA repair and redox activities, thereby blocking the transcription of genes essential for tumor cell survival, growth, and angiogenesis [11–16, 59, 60]. The dual nature of APE1/Ref-1 could promote repair of damage on promoter sites, possibly incurred in hypoxic tumor microenvironments, while simultaneously reducing transcription factors, thus ensuring proper transcription factor complex formation and gene expression (Figure 2). Therefore, simultaneous downregulation of transcription factors in cancer cells by inhibition of APE1/Ref-1 with dietary agents could decrease both cell survival and enhance tumor radio- and chemosensitivity.

Sensitization of tumor cells to radiation or chemotherapy by dietary agents could also effectively combat cancer by reducing the tumor burden while simultaneously relieving normal tissue toxicity, thus reducing the adverse effects of therapy. This hypothesis was supported by our clinical trial in prostate cancer patients showing that patients receiving soy isoflavones during and after radiation therapy showed better PSA level reduction and decreased incidence of urinary, gastrointestinal, and erectile dysfunction compared to those patients receiving placebo [100]. Our preclinical *in vitro* and *in vivo* studies suggest that the anticancer properties of soy isoflavones could be better exploited if these natural compounds are used as a complementary approach to conventional radiotherapy [59, 60].

The *in vitro* molecular effects of dietary agents on APE1/Ref-1 expression and activity need to be further studied *in vivo*. Our findings studying the effect of soy isoflavones on APE1/Ref-1, NF-κB, and HIF-1α demonstrated that these molecules truly represent potential biological targets for cancer therapies. Future research directions should include

elucidation of the molecular mechanisms of the differential effects of dietary agents acting as adjuvants for cancer cell therapy and as antioxidants for normal tissues. Further studies are warranted to determine the role of soy isoflavones, resveratrol, curcumin and the use of dietary antioxidants as chemo- and radioenhancers for tumors and radioprotectors for normal tissues in preclinical tumor models. This is particularly needed for critical cancer sites including lung, head and neck, and brain, sites in which treatment-induced injury to normal surrounding tissues result in serious early and late effects. Combination therapies for advanced cancers, including radiotherapy and chemotherapy, could benefit from a complementary and safe approach using dietary agents to mitigate the adverse effects of these therapies on normal tissues and are under active clinical investigation [10]. Elucidation of the mechanisms of interaction between dietary agents and conventional cancer treatments will have a strong impact on understanding the basic science of cancer chemoprevention and will justify the continued clinical use of dietary agents as an adjuvant to standard cancer treatment.

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Review Article

Potential Anticancer Properties of Grape Antioxidants

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Dietary intake of foods rich in antioxidant properties is suggested to be cancer protective. Foods rich in antioxidant properties include grape (*Vitis vinifera*), one of the world's largest fruit crops and most commonly consumed fruits in the world. The composition and cancer-protective effects of major phenolic antioxidants in grape skin and seed extracts are discussed in this review. Grape skin and seed extracts exert strong free radical scavenging and chelating activities and inhibit lipid oxidation in various food and cell models *in vitro*. The use of grape antioxidants are promising against a broad range of cancer cells by targeting epidermal growth factor receptor (EGFR) and its downstream pathways, inhibiting over-expression of COX-2 and prostaglandin E2 receptors, or modifying estrogen receptor pathways, resulting in cell cycle arrest and apoptosis. Interestingly, some of these activities were also demonstrated in animal models. However, *in vivo* studies have demonstrated inconsistent antioxidant efficacy. Nonetheless, a growing body of evidence from human clinical trials has demonstrated that consumption of grape, wine and grape juice exerts many health-promoting and possible anti-cancer effects. Thus, grape skin and seed extracts have great potential in cancer prevention and further investigation into this exciting field is warranted.

1. Introduction

Grape (*Vitis vinifera*) is one of the world's largest fruit crops. Grape is also one of the most commonly consumed fruits in the world both as fresh fruit (table grape) and processed fruit (wine, grape juice, molasses, and raisins) [1, 2]. There are greater than one hundred grape species which are divided into 2 subgenera: *Euvitis and Muscadinia*. Most of the species are in *Euvitis* subgenera [3]. Commercially cultivated grapes are usually classified as table or wine grapes depending on their intended method of consumption [4]. Table grapes usually bear large, seedless fruit berries with relatively thin skin, whereas wine grapes are smaller with thick skins [5]. Grapes are known to contain substantial amount of simple sugars especially glucose and fructose. For instance, wine grapes usually contain 19% or higher sugar content by fresh weight [6]. A growing body of epidemiological studies and randomized controlled human trials have associated the consumption of grapes, wine, and grape juice with a wide variety of health-promoting effects particularly the reduced risk of cardiovascular diseases, type-2 diabetes, certain types of cancers, and other chronic complications [7–13].

2. Phenolic Antioxidants in Grapes

The beneficial effects of grape and relevant grape-derived food products are believed to be related to a variety of bioactive components in grapes [14–16]. One major group of these components is phenolic antioxidants typically including anthocyanins, catechins, resveratrol, phenolic acids, and procyanidins [17]. Based on our analysis, 100 grams of fresh grapes contain 63–182 mg of the phenolic compounds [18]. Flavonoids constitute the majority of phenolic compounds (65–76%) in grapes. In red grapes, anthocyanins are the major group of the flavonoids [18]. Most of grape phenolic antioxidants are distributed in grape skins or seeds [19]. For instance, resveratrols, antochyanins, and catechins are concentrated in the skin part, while procyanidins are concentrated in grape seeds [20]. The commercial grape skin or seed extracts are made from grape pomace which is typically

regarded as a waste byproduct generated in the lucrative winemaking industry [21]. Large amounts of this byproduct accumulate annually which leads to a waste-management issue [22]. It is estimated that the harvested grapes will generate approximately 20% of grape pomace. However, uses of grape pomace are limited but have been recycled as organic fertilizers, manure, and animal feed [23]. Because grape skins and seeds are the predominant constituents in the pomace, this biomass is a rich source of phenolic antioxidants [20, 21]. We have previously extracted phenolic compounds from the skins of fresh Norton grapes which are wine grapes with unusual small sizes. The Norton grape skin contains 215.6 mg phenolic compounds per gram of the extract [24]. The most abundant components in grape skin are those flavonoids. The concentration of catechin and epicatechin is 8.71 and 3.45 mg per gram of the Norton skin extract [24]. Grape skin also contains a substantial amount of phenolic acids such as gallic acid, ferulic acid, caffeic acid, syringic acid, and p-coumaric acid with some being bound with sugars. Resveratrol is one of the most prominent bioactive components in grapes. Although resveratrol is predominantly contained in the grape skin, its concentration is only 0.21 mg per gram of the extract based on our HPLC analysis [24].

It is estimated that approximately 60–70% of grape polyphenols exist in grape seeds [25]. In contrast to grape skins, grape seeds contain a main unique group of phenolic compounds, procyanidins, which are flavan-3-ol derivatives and are colorless in the pure state [26]. Oligomeric and polymeric procyanidins in grape seeds possess a broad spectrum of pharmacological, medicinal, and therapeutic properties and are one of the most potent natural antioxidants [27–30]. They can be extracted during the latter stages of winemaking and are believed to contribute to color stability and organoleptic properties of wine [4–6]. In recent years, these proanthocyanidin compounds have extracted and purified from grape seeds and have become a common nutritional supplement. On the basis of structural characteristics, grape seed proanthocyanidins belong to condensed tannins. They mostly consist of (+) catechin and (−) epicatechin units, linked by C–4–C–8 or C–4–C–6 bonds and sometimes esterified by gallic acid on the epicatechin moiety(ies) [26]. These proanthocyanidins are the oligomer and polymer of flavan-3-ol with an average degree of polymerization (DP) ranging from 2 to >15 and an average molecular mass ranging from 578 to >5000 Da [1]. The structures of grape dimeric and trimeric proanthocyanidins have been recently elucidated [26]. The proanthocyanidin content in grape seeds is highly dependent on their varieties and extraction procedures. In general, the galloylated procyanidins are present in considerably lower concentrations than the nongalloylated ones [31], and higher molecular weight polymers constitute the majority of proanthocyanidins in grape seeds [26].

3. Antioxidant Properties of Grape Phenolic Compounds

Antioxidant activities of grape phenolic compounds have been extensively investigated *in vitro* and *in vivo*. Studies from us and others showed that grape skin, seed, and pomace extracts possess potent free radical scavenging activities using oxygen radical absorbance capacity (ORAC), 2,2-diphenyl-1-picrylhydrazyl (DPPH), and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS). Assays and some complex phenolics also show significant chelating activity on transition metal ions which are strong promoters of lipid peroxidation [18, 24, 27, 32]. The antioxidant activities of grape phenolics have also been demonstrated in various model systems such as protecting low-density lipoprotein (LDL) against oxidation brought about by Cu^{2+}, oxygen-centered radical-generating AAPH, or peroxynitrite-generating SIN-1 *in vitro* systems, preventing spleen cells from DNA damage induced by hydrogen peroxide (H_2O_2), and reducing oxidative stress in PC12 cells induced by addition of Fe^{2+} and *t*-butyl hydroperoxide [33–35]. However, the *in vivo* studies examining antioxidant activity of grape extracts have shown inconsistent results. Some studies showed that dietary intake of grape antioxidants helps to prevent lipid oxidation and inhibit the production of reactive oxygen species (ROS). For instance, dietary supplementation of grape seed extract (600 mg/day) for 4 weeks was shown to reduce oxidative stress and improve glutathione (GSH)/oxidized glutathione (GSSG) and total antioxidant status (TAOS) in a double-blinded randomized crossover human trial [9]. Another study also demonstrated that grape seed extract supplementation (2×300 mg/day) improved plasma antioxidant capacity in the high-cholesterol human subjects [36]. While other studies showed that dietary supplementation of grape juice, grape skin, or grape seed extracts exhibits either only a moderate antioxidative effect [11, 37, 38] or a neutral effect in animals and humans [39–41]. We have recently found that 3-month dietary supplementation of grape skin and grape pomace antioxidant extracts (0.2% in diet, equivalent to equivalent to approximately 960 mg GSE/day for humans) showed no effects on oxidative stress in diet-induced obesity mice [41, 42]. These results are consolidated by a recent report which showed that dietary supplementation of grape skin extract (1170 mg/day) had no significant effect on antioxidant enzymes including superoxide dismutase or catalase and also had no effect on 2-aminoadipic semialdehyde (AAS) residues, a plasma protein oxidation product, or on malondialdehyde in plasma or in LDL, markers of lipoprotein oxidation in physically active individuals [43]. This inconsistency may be related to the low absorption of grape phenolics since the absorption rate of polyphenol antioxidants is generally less than 1% [44].

4. Anticancer Properties of Grape Phenolic Compounds

Grape antioxidants have drawn an increased attention for their potential anticancer effects. A number of studies suggest that the high consumption of grape components could be associated with the reduced risk of certain cancers such as breast cancer and colon cancer [45–47]. The anticancer effects of grape antioxidants have been demonstrated in *in vitro* and *in vivo* models [48–52]. Grape antioxidants

have been shown to induce cell cycle arrest and apoptosis in cancer cells [53] as well as prevent carcinogenesis and cancer progression in rodent models [54, 55]. Considering the diversity of grape antioxidants, it is very likely that these compounds are to exert potential anticancer activity by acting on multiple cellular events associated with tumor initiation, promotion, and progression. Proposed mechanisms of potential anticancer effects of grape antioxidants include antioxidant, anti-inflammatory, and antiproliferative activities [56]. Grape antioxidants could act as free radicals scavengers, and chelating agents help to reduce physiological reactive oxygen species (ROS) [57]. ROS is known as an important mediator of apoptosis since initiation and regulation of apoptosis is associated with modifications in the oxidative environment [58]. Study has shown that dietary intake of grape antioxidants reduced rat mucosal apoptosis via modulation of both mitochondrial and cytosolic antioxidant enzyme systems together with an increase in cellular glutathione (GSH): glutathione disulfide (GSSG) ratio, protecting normal colonic mucosa from reactive oxygen species (ROS) attack [25, 59]. Grape antioxidants also exert anti-inflammatory activity which is believed to be associated with their chemopreventive effects [25, 41, 42]. Cancer chemopreventive agents include nonsteroidal anti-inflammatory drugs (NSAIDs) such as indomethacin, piroxicam, and sulindac, all of which inhibit cyclooxygenase (COX) [60]. This inhibitory activity is relevant to cancer chemoprevention because COX catalyzes the conversion of arachidonic acid to proinflammatory substances such as prostaglandins, which can stimulate tumor cell growth and suppress immune surveillance [60, 61]. Moreover, COX may activate carcinogens to more reactive substances that cause genetic damage [62]. A number of studies have demonstrated the inhibitory effects of whole grape extracts, individual grape antioxidants, or their mixture on COX activity and gene expression [63–65]. In addition, a study found that grape antioxidants exert an antitumor activity partially related to their immunopotentiating activities through the enhancements of lymphocyte proliferation, NK cell cytotoxicity, CD4+/CD8+ ratio, IL-2, and IFN-γ productions [66].

Grape antioxidants are also shown to modify estrogen receptor (ER) and are therefore especially relevant for gynecological cancers such as breast cancer [67]. For example, some grape antioxidants such as resveratrol, quercetin, and catechin exhibit both estrogenic and antiestrogenic effects due to their structural similarity to the steroid hormone estrogen [67, 68]. Resveratrol was reported to bind ERβ and ERα with comparable affinity but that the estrogen agonist activity of resveratrol was greater with ERβ than with ERα [69]. The binding affinity of grape skin anthocyanidins (anthocyanin aglycones) to ERα was 10,000- to 20,000-fold lower than that of the endogenous estrogen estradiol and these compounds at treatment levels of 10–20 μmols/L on MCF-7 cells exhibited a weak but statistically significant estrogenic activity [70]. However, in combination treatments with estradiol, three grape anthocyanidins showed antiestrogenic activity, which could be potentially explained by competition for the estrogen receptor and lower intrinsic activity of the phytoestrogens than estradiol [70]. It has been suggested that the number of hydroxy groups in grape pigments had a substantial effect on the estrogen activity. The presence of up to two hydroxy groups in the B-ring of the molecular structure decreased the affinity of the anthocyanidins to the ERα [71]. Grape anthocyanidins showed estrogen-inducible cell proliferation in MCF-7 breast cancer cell line but not in the receptor-negative MDA-MB-231 cell line. The fact that 4-hydroxytamoxifen, the receptor antagonist, can block the anthocyanidins-induced cell proliferation and combination treatments of anthocyanidins with estradiol-reduced proliferative activity of estradiol strongly suggest that the estrogenic activity of certain grape is relevant to its beneficial activity against estrogen-dependent cancers [70].

Epidermal growth factor receptor (EGFR) is a type I tyrosine kinase receptor belonging to a family of receptors that also includes HER2, HER3, and HER4. Aberrant EGFR activation, mediated primarily through changes in gene amplification and autocrine stimulation, appears to be a key factor in tumorigenesis, as well as an essential driving force for the aggressive growth behavior of cancer cells [72]. Grape antioxidants have been shown to inhibit expression of epidermal growth factor receptor (EGFR) in head and neck squamous cell carcinoma (HNSCC) cells which also caused an inhibition of the phosphorylation of extracellular signal-regulated kinase (ERK1/2), the highly conserved Ras/mitogen-activated protein kinase (MAPK)-dependent pathway (one of EGFR major downstream pathways) [57]. This effect is supported by another study that found that grape seed antioxidants inhibited constitutive activation of MAPK/ERK1/2 and MAPK/p38 in MDA-MB-468 cells [73]. The effects of grape antioxidants on cell cycle arrest are reported to be involved in promoting the expression of p21(Cip1)/p27(Kip1) protein G1-phase arrest [74]. Grape antioxidants can also target the transcription factor nuclear factor kappa B (NF-κB) by inhibiting its DNA-binding capacity to inhibit cancer cell invasion [75]. Recent *in vitro* and *in vivo* studies on potential anticancer properties of grape antioxidants are discussed as follows.

5. *In Vitro* Anticancer Properties of Grape Phenolic Compounds

Grape antioxidants especially grape seed proanthocyanidins (GSPs) show very promising inhibitory effects on a variety of cancer cells. Non-small-cell lung cancer (NSCLC) represents approximately 80% of total lung cancer cases, the leading cause of cancer-related deaths. GSPs have been shown to induce apoptosis of NSCLC cells: A549 and H1299, which are mediated through increased expression of proapoptotic protein Bax, decreased expression of antiapoptotic proteins Bcl2 and Bcl-xl, disruption of mitochondrial membrane potential, and activation of caspases 9, 3, and poly(ADP-ribose) polymerase (PARP) [76]. Overexpression of COX-2 and prostaglandins (PG) is linked to a wide variety of human cancers. *In vitro* treatment of NSCLC cells (A549, H1299, H460, H226, and H157) with GSPs resulted in significant growth inhibition and induction of apoptosis, which were associated with the inhibitory effects of GSPs

on the overexpression of COX-2 and prostaglandin (PG) E2 receptors (EP1 and EP4) in these cells [77].

Grape seed extract (GSE) was also found to selectively inhibit the growth and cause cell cycle arrest and apoptotic death in both Detroit 562 and FaDu HNSCC cells by activating DNA damage checkpoint cascade, including ataxia telangiectasia mutated/ataxia telangiectasia-Rad3-related-checkpoint kinase 1/2-cell division cycle 25C as well as caspases 8, 9, and 3 [78]. In addition, GSE-caused accumulation of intracellular ROS was identified as a major mechanism of its effect for growth inhibition, DNA damage and apoptosis, which was remarkably reversed by antioxidant N-acetylcysteine [78]. GSPs have recently shown to concentration-dependent inhibit human cutaneous HNSCC cell invasion, which was associated with a reduction in the levels of epidermal growth factor receptor (EGFR) and the inhibition of the phosphorylation of ERK1/2, a member of mitogen-activated protein kinase family [57]. Additionally, inhibition of human cutaneous HNSCC cell invasion by GSPs was associated with reversal of epithelial-to-mesenchymal transition (EMT) process, which resulted in an increase in the levels of epithelial biomarker (E-cadherin) while loss of mesenchymal biomarkers (vimentin, fibronectin, and N-cadherin) in cells [57]. These data suggest a potential for GSPs to be developed and used for the prevention of invasion/metastasis of HNSCC cells.

Melanoma is the leading cause of death from skin disease, and treatment of human melanoma A375 and Hs294t cells with GSPs resulted in a concentration-dependent inhibition of invasion or cell migration of these cells, which was related to a significant reduction in the levels of COX-2 expression and PGE(2) production [79]. GSPs *in vitro* are also effective on oral squamous cell carcinoma (OSCC). OEC-M1 cells lead to cell cycle arrest by increasing the expression of p21(Cip1)/p27(Kip1) protein without functioning mitochondria-mediated apoptosis, whereas GSP on SCC-25 cells inhibits cell proliferation via both G1-phase arrest and mitochondria-mediated apoptosis in a dose-dependent manner as a result of alterations of Bcl-2 [74]. Moreover, GSP can inhibit the migration and invasion of both cells, which are associated with the suppression of matrix metalloproteinases (MMPs), MMP-2, and MMP-9 [74].

Studies investigating the effect of grape products on breast cancer also show promising results. A polyphenolic fraction isolated from grape seeds that is rich in procyanidins inhibits constitutive activation of MAPK/ERK1/2 and MAPK/p38 and causes an induction of CDKI Cip1/p21 and a decrease in CDK4 in MDA-MB-468 cells [73]. Constitutive activation of ERK1/2 pathway has been shown to be associated with human breast carcinomas and derived cell lines for uncontrolled growth [80, 81]. These effects of GSP result in a G1 arrest in cell cycle, followed by an irreversible inhibition of cell growth [73]. The activity is supported by another study which found that GSE upregulates p21 (Cip1) through redox-mediated activation of ERK1/2 pathway and posttranscriptional regulation leading to cell cycle arrest in colon carcinoma HT29 cells [82].

GSE is also protective against prostate cancer which was shown to inhibit histone acetyltransferases (HATs) in LNCaP cells, leading to decreased androgen-receptor- (AR-) mediated transcription and cancer cell growth [83]. In addition, GSE can downregulate urokinase plasminogen activator (uPA) and DNA-binding activity of the transcription factor nuclear factor kappa B (NF-κB) in highly metastatic androgen-independent PC3 prostate cancer cells and therefore inhibits cell invasion [75]. Study found that procyanidins from wild grape seeds can regulate ARE-mediated enzyme expression via Nrf2 coupled with p38 and PI3K/Akt pathway in HepG2 cells and could be used as a potential natural chemopreventive agent through Nrf2/ARE-mediated phase II detoxifying/antioxidant enzymes induction via p38 and PI3K/Akt pathway [84]. Growth of certain colon cancer cells is also inhibited by GSE which exerts both antiproliferative and apoptotic effects on Caco2 and HCT-8 colon cancer cells, and its inhibitive effects were stronger than isolated procyanidins, suggesting a potential additive or synergistic effect among the grape seed components [85]. Another study found that the combination of resveratrol, a prominent grape skin component, and grape seed extract induces much more pronounced apoptosis in colon cancer cells, which is strongly correlated with p53 levels and Bax : Bcl-2 ratio [86].

6. *In Vivo* Anticancer Properties of Grape Phenolic Compounds

Most *in vivo* studies of anticancer properties of grape components focused on GSE or proanthocyanidin fraction of GSE (e.g., GSPs). In 1999, Agarwal and his colleagues conducted a thorough review on *in vivo* efficacy and potential working mechanisms of GSE and grape-based products against a variety of cancers [87]. Therefore, in this paper we discussed the *in vivo* studies that were conducted since 1999. Dietary intake of GSE (0.2% GSE wt/wt in diet) decreased HNSCC Detroit 562 and FaDu xenograft tumor growth by 67 and 65% ($P < 0.001$), respectively. Xenografts from GSE-fed groups showed decreased proliferation but increased DNA damage and apoptosis [78]. However, the exact molecular mechanisms are remained to be elucidated. Administration of 50, 100, or 200 mg GSPs/kg body weight of mice by oral gavage (5 d/week) markedly inhibited the growth of NSCLC A549 and H1299 lung tumor xenografts in athymic nude mice, which was associated with the induction of apoptotic cell death, increased expression of Bax, reduced expression of antiapoptotic proteins, and activation of caspase-3 in tumor xenograft cells [76], suggesting a consistency between the observations of *in vitro* and *in vivo* studies. The growth-inhibitory effect of dietary GSPs (0.5%, w/w) was also shown on the NSCLC xenografts, and the inhibition was associated with the inhibition of COX-2, PGE(2), and PGE(2) receptors (EP1, EP3, and EP4) in tumors [77]. GSE may also be effective in the prevention of certain types of cancers. A recent study showed that dietary supplementation of grape seed extract (0.25 or 0.5% (w/w) caused strong chemopreventive efficacy in Fischer 344 rats against azoxymethane- (AOM-)induced aberrant crypt foci (ACF) formation (as much as

60% reduction in number of ACF and 66% reduction in crypt multiplicity) [88].

The literature search on various databases found only one human trial on grape products and cancer treatment that examined the role of grape products specifically grape seed proanthocyanidin extract in patients with breast induration following radiotherapy for breast cancer and found no significant effect. With respect to preventive effect, a human study showed that 8-week dietary supplementation of grape juice (480 mL/day) reduced lymphocyte DNA damage by reducing the formation of reactive oxygen species by as much as 15% [81], suggesting that a potential anticarcinogenic role for grape products by providing antioxidant protection.

In summary, with respect to cancer prevention and treatment, grape seed extracts or its proanthocyanidins have received most investigation for their potential anticancer activities. Some studies show very promising potential of GSE as an anticancer agent. However, most available reports are focusing on their *in vitro* activities and mechanisms. Since the absorption of these phenolic compounds is limited, the dose responses of GSE and its components need to be determined in animals and humans as well as its *in vivo* mechanisms. The potential use of grape skin and seed extracts in cancer prevention has great potential, and further investigation into this exciting field is warranted.

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Review Article

Potential Role of Garcinol as an Anticancer Agent

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Garcinol, a polyisoprenylated benzophenone, is extracted from the rind of the fruit of *Garcinia indica*, a plant found extensively in tropical regions. Although the fruit has been consumed traditionally over centuries, its biological activities, specifically its anticancer potential is a result of recent scientific investigations. The anticarcinogenic properties of garcinol appear to be moderated via its antioxidative, anti-inflammatory, antiangiogenic, and proapoptotic activities. In addition, garcinol displays effective epigenetic influence by inhibiting histone acetyltransferases (HAT 300) and by possible posttranscriptional modulation by mi RNA profiles involved in carcinogenesis. *In vitro* as well as some *in vivo* studies have shown the potential of this compound against several cancers types including breast, colon, pancreatic, and leukemia. Although this is a promising molecule in terms of its anticancer properties, investigations in relevant animal models, and subsequent human trials are warranted in order to fully appreciate and confirm its chemopreventative and/or therapeutic potential.

1. Introduction

The extract from the fruit of *Garcinia indica*, popularly known as *Kokum* or *Mangosteen* has been valued in the Indian subcontinent, Africa, and China for its sweet and sour taste and has traditionally been used as a seasoning, a snack, or steeped in syrup for a refreshing drink. In addition, it has been recommended by the Ayurvedic system of medicine for treatment of ailments such as heat strokes, infections, and edema [1].

The major chemical constituents of the fruit extract include citric acid, hydroxycitric acid (HCA), hydroxycitric acid lactone, and oxalic acid in addition to the benzophenone derivatives, garcinol, and its isomer isogarcinol [1]. Of these, HCA has shown hypocholesterolemic and antiobesity activity, while the benzophenone derivatives have been associated more closely with antioxidant behavior. In this paper, we will concentrate on the biological properties of garcinol with a specific focus on its anticarcinogenic capabilities. Figure 2 gives an overview of the mechanistic insight into the anticancer activity of garcinol.

2. Chemical Properties of Garcinol

Garcinol ($C_{38}H_{50}O_6$; molecular weight 602), a yellow crystalline compound, with a melting point of 132°C is structurally similar to curcumin, an established antioxidant, antiaging, and anticarcinogenic agent, by virtue of containing both phenolic hydroxyl groups and the β-diketone moiety [2].

The chemical structure of garcinol, also known as Camboginol (Figure 1), was elucidated by Rao and coworkers in 1980 using proton NMR and IR spectroscopy where they determined the location of a terminal alkene as well as the presence of a β-diketone from the compound's ability to enolize [2]. The terminal alkene contained in an unsaturated isoproprenyl substituent of the molecule can undergo cyclization under acidic conditions to produce isogracinol [3]. The latter, oxidized isomer of garcinol has also been associated with antitumorigenic, antiobesity, antiulcer, antibacterial, antiviral, and anti-inflammatory properties [4]. The chemical structure of garcinol is similar to that of a series of naturally occurring phytochemicals such as

the chalcones, oglogifolins, and Guttiferones [5, 6]. Liquid chromatography-tandem mass spectrometry method to rapidly and sensitively detect the presence and quantify the amount of garcinol present in a plant extract has now been developed, paving the way to a rapid source of Garcinol and hence a higher propensity of scientific investigations in this area [7].

The chemistry of garcinol and its synthetic chalcone analogs has been detailed in an earlier review [1]. In brief, as compared to the native chalcones fluorinated 2 hydroxychalcones have been reported to display increased antioxidative potential and bioavailability, which was exhibited effectively in pancreatic (BXPC 3) and breast cancer (BT-20) cell lines [8].

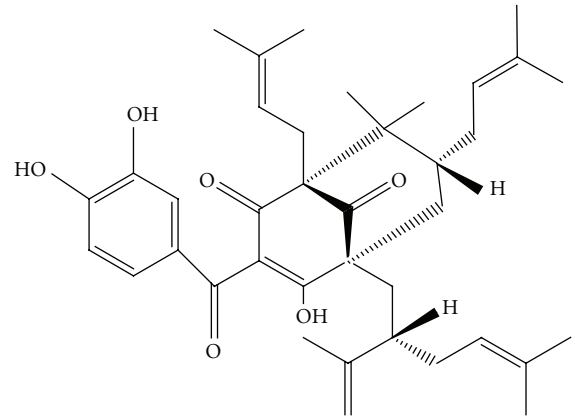

Figure 1: Chemical structure of Garcinol [15].

3. Antioxidative Properties of Garcinol

Oxidation and inflammation are considered to be underlying common causes of many chronic diseases including atherosclerosis, Alzheimer's' disease, cancer as well as the normal process of aging. Specifically, oxidative stress, the imbalance between a system's oxidative and the antioxidative pathways, in favor of the former, is brought on primarily by free radical species, resulting in cellular damage [9]. Antioxidants, by definition, inhibit oxidation by either inhibiting pro-oxidants, thereby preventing the formation of free radical species or by retarding the rate of reaction of oxidative species with their biological targets, thereby slowing down the free radical chain propagation [9]. Since the free radical chain, if allowed to propagate, can follow multiple pathways leading to a vast number of end products, measurement of oxidative stress or a substance's antioxidative ability has always been considered to be precise, relative to a specified biological condition or status, at best. Owing to the large number of molecules affected by free radicals, leading to a varying population of end products, a sizable array of independent assays have been put forward for the measurement of oxidative stress or an agent's antioxidative capacity.

The antioxidative ability of garcinol has been investigated in *in vitro* and *in vivo* model systems [10]. Using a hypoxanthine/xanthine oxidase system, garcinol was shown to retard superoxide anion to nearly the same amount as DL-alpha-tocopherol, an established anti-oxidant, while its ability to quell hydroxyl radicals in the Fenton reaction system was even better than that of alpha-tocopherol. In addition, the authors also explored the antioxidative power of garcinol *in vivo* using an indometacin induced rat model for acute ulceration. Oral administration of garcinol prevented acute ulceration in these rats, suggesting its potential as an antiulcer drug [10]. In another study, glutathione levels, known to be depleted due to oxidative stress in the erythrocytes of streptozotocin-induced type-2 diabetic rats, were effectively restored by oral administration of an aqueous extract of the fruit of *Garcinia indica* [11]. This could be attributed to the anti-oxidant potential of garcinol in the fruit extract. The protective role of garcinol in neuronal survival and differentiation was investigated in cultured cortical progenitor cells. [12]. In another study, antioxidative and neuroprotective properties of garcinol in rat cortical neuron cultures was observed. This was suggested to occur via prevention of nitric oxide NO accumulation in lipopolysaccharide treated astrocytes [13].

The examples above help to establish the antioxidative effect of garcinol *in vitro* and *in vivo* model systems. In terms of its structure, the phenolic hydroxyl groups coupled with the β-diketone moiety in garcinol may, via formation of resonance stabilized intermediates, help to prevent the free radical species from propagating and thereby limiting further oxidative-stress-related damage down the road.

4. Anticancer Properties of Garcinol

Cancer, the uncontrolled growth and spread of abnormal cells, may be initiated due to multiple factors including exposure to carcinogens, repeated genetic damage, by oxidative stress, chronic inflammation, or hormonal imbalance. Once initiated, a cascade of reactions ensues, making it difficult to specify molecular targets for therapeutic advancements. As such, most chemotherapeutic treatments suffer from adverse toxic reactions leading to acute and delayed nausea, mouth ulcerations, fatigue, nerve damage, blood clots, anemia, and mild impairments [14]. Thus, it is important to develop effective preventative and/or therapeutic approaches, which could potentially be both effective against cancer cell growth and relatively nontoxic. Recent evidence supports that nonnutritive components in diet have therapeutic benefits attributable to their pleiotropic effects including downregulation of survival signaling and simultaneous activation of multiple death pathways in cancer cells.

A number of recent studies have examined the potential of garcinol, a non-nutritive dietary component, against different cancer types (Table 1). In the process, a number of possibilities that explain the underlying mechanism for the chemopreventative and/or therapeutic performance of garcinol have also arisen. These are summarized below.

4.1. Garcinol: Effect on Inflammatory Pathways. The effect of dietary garcinol on the development of azoxymethane-

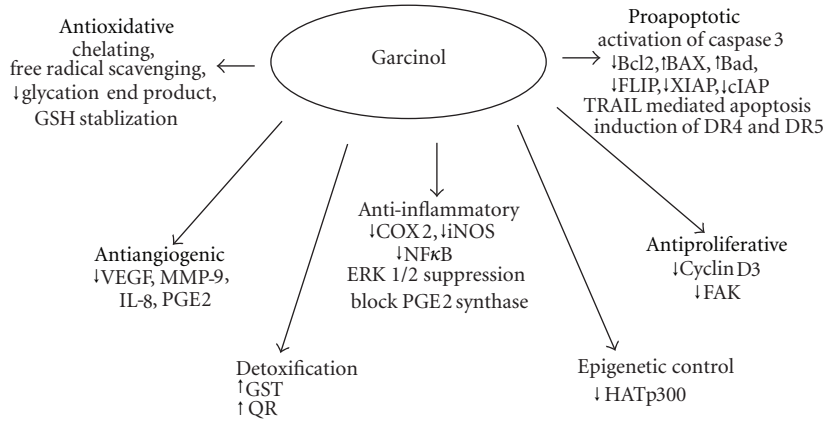

Figure 2: Anticancer activity of Garcinol: mechanistic targets.

Table 1: List of studies investigating the role of Garcinol in different cancer types.

Type of cancer	Study type	Experimental details	Reference
Breast cancer	*In vitro*	MDA, MB231, MCF-7	[16]
	In vitro	MCF-7, MDA-MB231	[17]
	In vitro	Primary culture, MCF-7 MDA-MB231, AU 565 BT-483	[18]
Burkitt lymphoma	*In vitro*	TPA induced Ebvirus-EA activated raji cells	[19]
Colon cancer	*In vitro*	HT-29, HCT-116	[16]
	In vitro	HT-29, HCT-116, ICE-6	[20]
	In vitro	HT-29	[21]
	In vivo	Azoxymethane induced colonic aberrant crypt foci F344 rats	[15]
Esophageal cancer	*In vitro*	SEG-1	[16]
Hepatocellular carcinoma	*In vitro*	MH1C1-HEP G2	[22]
	In vivo	Hep3B	[23]
	In vitro	Carcinogen induced liver cancer F344 rats	[24]
HeLa cells	*In vitro*	HeLa core Histones-P300 HAT inhibition	[25]
	In vitro	HeLa Histones	[26]
Kidney cancer	*In vitro*	A293 (Human embryonic kinder carcinoma)	[16]
Leukemia	*In vitro*	KBM-5 (chronic Leukemia)	[16]
	In vitro	U937, K562, NB4, HL60	[27]
	In vitro	HL-60	[28]
	In vitro	HL-60	[15]
Lung cancer	*In vitro*	A-549	[29]
Medulloblastoma	*In vitro*	Daoy, growth factor,	[24]
Multiple myeloma	*In vitro*	U-266	[16]
Pancreatic cancer	*In vitro*	BxPC3	[30]
	In vitro	Panc-1, BxPC-3	[31]
Prostate cancer	*In vitro*	LNCaP, C4-2B, PC-3	[30]
	In vitro	PC-3	[16]
Tongue cancer	*In vivo*	4-nitroquinoline 1-oxide induced oral carcinogenesis in F344 rats	[32]

(AOM-) induced colonic aberrant crypt foci (ACF), precursors for colon cancer, was investigated in male F344 rats [15]. Ingestion of 0.01% and 0.05% dietary garcinol in rat models significantly reduced the formation of ACF in a dose-dependent manner, thus suggesting suppression in cancer development [15]. No adverse effects were reported with diets containing up to 0.05% by weight of garcinol [15]. Additionally, garcinol consumption provided the added benefit of increased liver glutathione S-transferase (GST) and quinone reductase (QR) levels, both detoxifying enzymes associated with cancer suppression [15, 33].

Since carcinogenesis is often accompanied with increase in oxidative stress, the authors also probed oxidative and inflammatory pathways and investigated the response on O2-, Nitric Oxide (NO), iNOS, and COX2 activity upon treatment with garcinol *in vitro*. Garcinol inhibited O2-, Nitric Oxide (NO), iNOS, and COX2 at a slightly greater level than the green tea polyphenol, epigallocatechin gallate (EGCG), an established plant antioxidant, in the cell lines evaluated [15]. Taking the two together, it is possible that the anticolon tumorigenesis activity of garcinol may in part be due to modulation of iNOS and COX2 activities, although mechanistic details need further elucidation [15].

The COX-2 expression in tongue lesions induced with 4-nitroquinoline 1-oxide (4-NQO) in male F344 rats was also decreased on feeding garcinol to these animals [32]. In this study, dietary garcinol was administered either during the period of exposure to the carcinogen or after the exposure to carcinogen was complete. The animals fed the garcinol-containing diet had significantly decreased the incidence and multiplicity of 4-NQO-induced tongue neoplasms and/or preneoplasms as compared to the control diet under both conditions. This shows the potential of garcinol both as a preventative and therapeutic agent against tongue cancer. In addition, the authors did not observe adverse effects with respect to survival rate, or any histological changes in the liver or kidney that could be related to toxicity.

In another study, the mechanisms by which garcinol modulates arachidonic acid metabolism and NO synthesis in lipopolysaccharide- (LPS-) stimulated RAW264.7 murine macrophages and in intestinal cell lines were examined [34]. Treatment with garcinol decreased the release of arachidonic acid and its metabolites in the cell lines tested. Results indicate that the suppression of arachidonic acid on treatment with garcinol after the activation of LPS-stimulated macrophages may occur via inhibition of $cPLA_2$and ERK 1/2, key factors in the release of arachidonic acid from membrane phospholipids [34]. Conversely, garcinol treatment prior to LPS-stimulation may lead to inhibition of the toll-like receptors upstream of ERK 1/2 [34]. Additionally, downregulation of NF-κB and COX-2 expressions, both involved in the inflammatory response were also observed [34].

Koeberle and coworkers showed that garcinol interferes with 5-lipoxygenase and microsomal prostaglandin PGE2 synthase enzyme activity, both of which play crucial roles in inflammation [29]. Further, garcinol inhibited synthesis of PGE2 and 5-lipoxygenase in human neutrophils and interleukin-stimulated human lung carcinoma cells [29]. The authors established that 5-lipoxygenase and certain prostanoids are possible targets of garcinol, and may be of potential pharmacological interest [29].

Prostaglandin E2 (PGE2) is produced by the action Cox2 enzyme. It has been documented that aggressiveness of pancreatic cancer increases with increase in the level of PGE2 [35]. Targeting PGE2 can decrease inflammation and proliferation and can reduce metastasis of pancreatic cancer through various factors like MMP-2 and MMP-9 [36, 37]. In our laboratory, we have observed significant reduction in PGE2 expression levels in Cox 2-containing pancreatic cancer cell line BXPC3 with treatment with garcinol.

Taken together, these studies demonstrate that inhibition of inflammatory pathways via multiple but related targets may play a pivotal role in garcinol's response as an anti-inflammatory and anticarcinogenic agent.

4.2. Garcinol: Effect on Apoptotic Pathways. Another approach for halting cancerous growth involves targeting apoptosis, programmed cell death of rapidly dividing cancer cells. Garcinol exhibited greater induction of apoptosis than curcumin in human leukemia HL-60 cells [28]. This was mediated by release of cytochrome c into the cytoplasm of the cell from the mitochondria allowing for the activation of caspase-3. Apoptosis was also accompanied with down-regulation of Bcl-2, an antiapoptotic protein and significant upregulation of Bad and Bax, established proapoptotic proteins. The results suggest that apoptosis affected by garcinol is initiated by the release of cytochrome c into the cytosol, followed by procaspase-9 processing, activation of caspase-3 and caspase-2, degradation of PARP, and DNA fragmentation [26–28].

Interestingly, when Hong and coworkers investigated the effect of garcinol on colon cancer and normal immortalized intestinal cell line, garcinol showed a much higher potency of inhibition against the colon cancer cells as compared to the normal cells. Greater growth inhibition was accompanied by increase in Caspase 3 activity, indicative of an apoptotic pathway [30]. This differential response in activity against cancer cells versus normal cells was also observed in breast cells [17, 20]. However, at exceptionally low doses ($<1\,\mu$M), garcinol was reported to stimulate intestinal cell growth, possibly due to production of hydrogen peroxide in the system, which may not be effectively quenched by the very low concentrations of garcinol [20]. Thus, the beneficial attributes of garcinol are dose dependent. This phenomenon of induction of cell growth at very low levels has been observed for other bioactive compounds as well including (−) epigallocatechin gallate [22].

Garcinol has also been shown to inhibit breast, prostrate and pancreatic cancer cell growth by Induction of apoptosis which was mediated by caspase-3 followed by downregulation of the NFκB pathway [17, 30]. Chen et al., however, documented that garcinol may exert its anticarcinogenic effects in nicotine-induced breast cancer cells by induction of cell cycle arrest at the G0/G1 stage of mitosis [18]. In addition, this group showed that garcinol achieved inhibition

of breast cancer cell proliferation by suppressing nicotine-induced nicotinic acetylcholine receptor (α9-nAChR) protein expression and downregulating type D cyclins associated with mitotic cell cycle arrest [18].

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a cytokine known to elevate apoptosis via death receptors DR4 and DR5, via their interaction with Fas-associated domain (FADD), subsequently leading to activation of the caspase-3 pathway. The aforementioned cytochrome c/caspase 3 pathway can also be initiated by TRAIL by mitochondrial cleavage and capase 9 activation [16]. On treatment with garcinol, various cancer cell lines (colon, prostate, breast, kidney, leukemic, esophageal) exhibited increased apoptosis via an induction of death receptors DR4 and DR5 [16]. Thus its action does not appear to be specific to a particular cancer cell type. Moreover, garcinol was also reported to suppress the expression of prosurvival proteins survivin, bcl-2, XIAP, and cFLIP and conversely amplified proapoptotic pathways such as inducing cell membrane cleavage, increasing bax expression, and subsequent release of cytochrome c [16].

Platelet-derived growth factor receptors (PDGFRs) are implicated in medulloblastoma, the most common brain tumor of childhood. A recent report detailed the cytotoxic and antiapoptotic effect of garcinol against brain tumor cells (Daoy). The cytotoxicity, specifically against PDGFR-regulated Daoy cells, was accompanied with a significant S phase cell cycle arrest, downregulation of cyclin A and E, and activation of caspases [38].

4.3. Garcinol: Effect on Epigenetic Control. Alteration in genetic expression is yet another mechanism for the onset of cancer. Hokaiwado et al. investigated changes of gene expression in livers of rats treated with carcinogens and tumor promoters using a novel, three-dimensional microarray system, customized to meet experimental requirements. Using the customized microarray, garcinol was classified as chemoprotective against liver cancer at a concentration of 0.05% [24]. It was suggested that the likely mechanism of garcinol's anticarcinogenic activity involved the suppression of histone acetyltransferases [26]. Histone acetylation is a mode of chromatin remodeling, which enables gene transcription there by altering cell growth [26]. Histone acetylation and deacetylation is enabled by histone acetyltransferases (HATs) and histone deacetyltransferases (HDACs) [26]. Dysfunction of HAT can lead to cancers and therefore inhibitors of HAT may be of use in cancer therapeutics [22, 25, 26]. Garcinol inhibits HATs p300 and PCAF, thereby suppressing cell proliferation, which leads to cancer repression [26]. Interestingly, the specific inhibition of p300-HAT by a garcinol derivative, LTK-14, resulted in a noncytotoxic inhibition of HIV proliferation by preventing acetylation of HIV-infected cells [39].

Micro-RNAs (miR) are a family of highly conserved short RNA products (17–25 nucleotides) that control gene expression at the posttranscriptional levels regulating different cancer types. Although the functional roles of all miRs are not completely understood, they may act as oncogenes and/or tumor suppressors regulating cellular differentiation, proliferation, and apoptosis. We evaluated the effect of garcinol alone and in combination with gemcitabine on microRNA profile in human pancreatic cancer cell lines, BxPC-3 and Panc-1. MicroRNA microarray profile revealed a variation in expression of several microRNAs. Interestingly in our study, miR-21 (a signature of tumor aggressiveness) was significantly downregulated on treatment with garcinol as compared to gemcitabine or the combination treatment in gemcitabine-resistant cell line Panc-1, suggesting its role in acquisition of chemoresistance. (unpublished data).

4.4. Garcinol: Effect on Proliferation, Angiogenesis, and Metastasis. Angiogenesis and metastasis are hallmarks of advanced stages of carcinoma. Control at these stages is crucial for patient prognosis. Garcinol has shown very promising antiangiogenic and antimetastatic activities *in vitro*. Studies from our laboratory, demonstrate that garcinol downregulated MMP-9, IL-8, PGE-2, and VEGF, markers of angiogenesis and metastasis in pancreatic cancer cell lines, Panc 1 and BxPC3. The effects of garcinol were even more pronounced in the Panc1 cell line, carrying the *k-ras* mutation, implicated in the majority of human pancreatic cancer patients [31]. In another study, the effect of garcinol and other dietary phytochemicals on cell proliferation and migration was examined in rat liver bioassays and human hepatic cell lines. Cell invasion assays using Matrigel analysis showed a decrease in hepatocyte growth factor- induced cell invasion of HepG2 and MH1C1 cells by garcinol. However, the effects were not statistically significant and may need further investigation. According to recent study done on dysfunctional P53 cell line Hep 3B Garcinol has shown induction of ROS dependant apoptosis through death receptor, mitochondrial and modulating GADD153 [22, 23].

We have also explored the possible synergistic effects between garcinol and other phytochemicals, including curcumin as well as garcinol and currently available cancer therapeutics, including gemcitabine, in pancreatic cancer cell lines (unpublished data). In brief, using a combinatorial design, our results showed that garcinol and curcumin in concert show a greater decrease in cell growth via increased induction of apoptosis in pancreatic cancer cells, Panc 1 and BxPC3 than either agent alone.

In combination with the therapeutic, garcinol was seen to synergistically sensitize pancreatic cancer cells to gemcitabine *in vitro*. Human pancreatic cancer cell lines, BxPC-3 and Panc-1, harboring wild and mutant *K-ras* genotypes, respectively, were treated with garcinol and gemcitabine individually and in combination at different doses and times of incubation, to monitor growth inhibition and degree of apoptotic cell death. The combination of garcinol and gemcitabine showed a significant reduction in cell growth and increase in apoptosis as compared to respective individual treatments. Further, garcinol in synergism with gemcitabine induced its action by downregulating NF-κB, VEGF, Il 8, *MMP-9* and activating PARP cleavage. This further highlights the potential of garcinol in combinatorial therapeutic strategies.

Garcinol has also been shown to be involved in the genetic modification of Focal Adhesion Kinase (FAK), a key player in the regulation of major processes in the cell including cell proliferation, migration, and apoptosis [21]. Garcinol was shown to downregulate FAK activity by blocking its phosphorylation [21]. Subsequently, this inhibition was shown to alter the Bcl-2/BAX ratio, further leading to the activation of caspase-3 at a dose of 20 μM [21].

5. Summary

Cancer, the uncontrolled growth and spread of abnormal cells, results from the accumulation of numerous sequential mutations and alterations in nuclear and cytoplasmic molecules [40]. Cancer progression is considered to involve three key steps: initiation, in which a normal cell is transformed into an initiated or abnormal cell, promotion, by which the initiated cell is converted into a preneoplastic cell, and progression, the process whereby the cells become neoplastic [41]. Cancer may be initiated due to multiple factors including exposure to carcinogens, repeated genetic damage by oxidative stress, chronic inflammation, or hormonal imbalance. This followed by a cascade of reactions, triggered by multiple signaling molecules makes it difficult to target a specific molecule responsible for the disease and thereby retard progression. Thus, to reduce cancer incidence and mortality rate and improve the survival time of cancer patients, new techniques and approaches must be developed to diagnose, prevent, and treat preinvasive lesions. Most chemotherapeutic treatments suffer from adverse toxic reactions leading to acute and delayed nausea, mouth ulcerations, fatigue, nerve damage, blood clots, anemia, and mild impairments [14]. Thus, it is important to develop effective preventative and/or therapeutic approaches either in the form of single agents or as combinations, which could potentially be both effective against cancer cell growth and relatively nontoxic. Recent evidence supports that nonnutritive components in diet have therapeutic benefits attributable to their pleiotropic effects including downregulation of survival signaling and simultaneous activation of multiple death pathways in cancer cells.

Here, we have attempted to present the potential preventative and/or therapeutic role of garcinol, the active component of *Garcinia* species against cancer progression. Although still preliminary in nature, recent evidence demonstrates that garcinol possesses multifunctional bioactivities including antimicrobial, anti-inflammatory, antioxidant, apoptotic, antitumorigenic, and perhaps anti-neurodegenerative as well.

Inflammation and oxidative stress are the key culprits implicated in numerous diseases, specifically in chronic conditions such as cancer, cardiovascular, and Alzheimer's disease. *In vitro* and some *in vivo* studies have shown that garcinol may inhibit these harmful chronic conditions from possibly manifesting and propagating via multiple mechanisms and sites of action. For instance, it behaves as a potent antioxidant with appreciable free radical scavenging activity, as also helping to avert inflammation, by suppressing proinflammatory signaling molecules, prostaglandins, leukotrienes and the signal transduction factor, NF-κB.

Garcinol's inhibition of cancer growth of various types including pancreatic, prostate, breast, leukemia, colon, and its progression at different stages, is especially intriguing. Nonetheless, the full potential of this compound has yet to be elucidated. Research-based evidence pointing to the noncytotoxic nature against normal cells, combined with potent proapoptotic behavior against cancerous cells, and its antimicrobial effects may be a testament to the traditional use of the plant against multiple ailments. Forthcoming data on its synergistic effect with known drugs at subtherapeutic doses may also open up new avenues for efficient therapeutic regimens without or with minimal adverse side effects. In addition, to date, no toxic effects of Garcinol have been reported, even when given orally upto 0.05% in diet. However, further investigation to evaluate the range of its therapeutic potential is required. Most of the advances in the anticancer effects of garcinol, although mechanistically exciting, have been as a result of *in vitro* studies. These may or may not be entirely reflected in an *in vivo* animal model or a clinical situation. Thus the studies at present, although potentially very useful, call out for an immense need for carefully planned and executed studies in relevant animal models of various cancer types to confirm these findings.

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Review Article

Lycopene, Tomato Products, and Prostate Cancer Incidence: A Review and Reassessment in the PSA Screening Era

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Lycopene has been proposed to protect against prostate cancer through various properties including decreased lipid oxidation, inhibition of cancer cell proliferation, and most notably potent antioxidant properties. Epidemiologic studies on the association between lycopene and prostate cancer incidence have yielded mixed results. Detection of an association has been complicated by unique epidemiologic considerations including the measurement of lycopene and its major source in the diet, tomato products, and assessment of prostate cancer incidence and progression. Understanding this association has been further challenging in the prostate-specific antigen (PSA) screening era. PSA screening has increased the detection of prostate cancer, including a variety of relatively indolent cancers. This paper examines the lycopene and prostate cancer association in light of epidemiologic methodologic issues with particular emphasis on the effect of PSA screening on this association.

1. Introduction

Several chemoprotective properties of lycopene on prostate cancer have been proposed, including potent antioxidant properties, decreased lipid oxidation, inhibition of cancerous cell proliferation at the G0-G1 cell cycle transition, and protection of lipoproteins and DNA [1, 2]. These mechanistic studies have stimulated the examination of lycopene and its primary source, tomato products, on risk of prostate cancer. However, studies on lycopene and tomato intake and prostate cancer incidence have yielded mixed results. The study of this relationship has been complicated by unique and challenging epidemiologic considerations in the measurement of lycopene and on the influence of prostate-specific antigen (PSA) screening on prostate cancer incidence and progression. As with all epidemiologic studies, validity depends on the quality of the methodology. This paper briefly describes the methodology essential for conducting studies on the association between lycopene and prostate cancer incidence and provides an updated review of studies of lycopene and tomato products with prostate cancer risk.

2. Measurement of Lycopene and Tomato Products

Lycopene is a carotenoid devoid of vitamin A activity. The major source by far, particularly in Western populations, is tomato and tomato products; a few other foods such as watermelons and pink grapefruit also contain lycopene. In epidemiologic studies, approaches to assess an individual's lycopene intake or status include studies that estimate intake of lycopene (based on reported intake of foods and food content of lycopene from food composition databases), studies that assess tomato product intake as a surrogate of lycopene intake, and studies that measure lycopene levels in the serum or plasma. An issue that is not unique for lycopene, but perhaps of special importance for this carotenoid, is the variable absorption of lycopene from different food sources. In particular, cooking in an oil medium substantially enhances bioavailability of lycopene in the intestine because lycopene is highly bound to plant source matrices and is highly lipophilic. The measure of lycopene in the serum has theoretical advantages of accounting for absorption and

not relying on study participants' food recall and accuracy of food composition tables. On the other hand, serum studies have frequently relied on a single measure, and how well a single measurement reflects long-term intake is not entirely clear. Also, since lycopene comes largely from tomato sources, circulating lycopene level may be acting as a surrogate of tomato product intake, and other components of tomatoes may account for any observed association with cancer risk. Finally, a population that consumes overall low levels of lycopene or similar levels of lycopene across individuals may result in insufficient contrast between high and low consumers.

3. The Influence of PSA Screening on Clinical and Epidemiologic Aspects of Prostate Cancer Incidence

PSA release into the serum occurs with tissue breakdown between the prostate gland lumen and capillaries. The original purpose of the PSA measurement was to monitor prostate cancer progression and recurrence. The Food and Drug Administration approved PSA testing for monitoring disease status in men with prostate cancer in 1987 and expanded its use to diagnosing prostate cancer in 1992. This approval was followed by professional society guidelines that supported the use of PSA testing for prostate cancer screening. Consequently, PSA became widely used as a screening test in the United States and increasingly in other countries. As a screening modality, the sensitivity and specificity of PSA varies based on the cut-off. For example, a PSA level of 3 ng/mL has a sensitivity of 32% for detecting any prostate cancer and 68% for high-grade prostate cancer and a specificity of 85%. If this level were increased to 4 ng/mL the sensitivity would decrease to 21% for any prostate cancer and 51% for high grade prostate cancer but specificity would improve to 91% [3].

Elevated serum PSA may precede invasive carcinoma by a minimum of 5–10 years. Thus, PSA testing enabled earlier detection of prostate cancer. The rate of first-time PSA testing was strongly correlated with prostate cancer incidence rates. With the onset of PSA for screening, prostate cancer incidence increased and peaked in 1992 and declined thereafter [4]. Localized, nonmetastatic cancers accounted for most of the increased incidence. Prior to widespread PSA use for screening, prostate cancer diagnosis was largely prompted by physical exam findings of an enlarged prostate or symptoms ranging from urinary incontinence to more advanced spinal cord compression and bony pain from metastasis; therefore, prostate cancer was mostly detected in relatively advanced stages. Invasive carcinoma prevalence increases with age: 2% for men in their 30 s compared with 64% for men in their 70s [5]. One-third of men under age 80 will have prostate cancer detected on autopsy. The lifetime risk of prostate cancer is 16% while the risk of mortality from prostate cancer is 2.9% [6]. Thus a "PSA screening era" beginning in 1988, FDA approved in 1992, and peaking between 1992 and 1998 has been referred to as a period in which prostate cancers diagnosed by serum PSA alone encompassed a variety of stages of prostate cancers, including clinically indolent cancers [4, 7].

PSA testing revolutionized the detection of prostate cancer but was not without unexpected consequences. In addition to diagnosing biologically indolent cancers, PSA elevation occurs with benign conditions including benign prostatic hypertrophy, prostatitis, subclinical inflammation, ejaculation, digital rectal exams (potentially performed just prior to patients having their PSA lab drawn), perineal trauma, prostatic infarction, urinary retention, biopsy, and transurethral resection of the prostate. The number of false positives is high, leading to numerous negative biopsies.

The influence of PSA on prostate cancer mortality has been controversial, with randomized trials not yielding a clear answer. However, undoubtedly PSA screening has caused an increase in the number of indolent cancers being treated aggressively and ultimately led to increased morbidity from side effects of treatment. The majority of newly diagnosed prostate cancers were clinically localized and unlikely clinically significant to involve aggressive medical and surgical therapy such as radical prostatectomy with radiation ablation intended to cure early-stage cancers. For these reasons, in 2011 the United States Preventive Services Task Force recommended against PSA screening for prostate cancer regardless of age, race/ethnicity, and family history.

Beyond the clinical consequences, PSA screening has altered the landscape of prostate cancer epidemiology. Many more cancers are diagnosed, including a substantial proportion of relatively indolent cancers, and the cancers are diagnosed earlier in their natural history, often before evidence of aggressive behavior is manifested. Thus, depending on at what stage and on what subtype of prostate cancer a risk factor may be acting, the relationship between this risk factor and prostate cancer risk may differ in populations exposed or not exposed to widespread PSA screening [8]. By increasing the heterogeneity in prostate cancers being diagnosed, PSA screening has added complexity to the epidemiologic study of prostate cancer.

4. Epidemiologic Studies

4.1. Clinical Trials. A number of randomized clinical trials have examined lycopene and prostate cancer progression and mortality in men diagnosed with prostate cancer [9]. The randomized studies have been small and inconclusive [10]. In a double-blind randomized placebo-controlled trial, 105 African American male veterans recommended for biopsy to detect prostate cancer were administered tomato sauce containing 30 mg/day of lycopene or placebo over 21 days [11]. PSA and lycopene levels were measured, and the group randomized to lycopene had an increase in serum lycopene and decrease in PSA while the placebo group had the reverse, with a decrease in serum lycopene and increase in PSA. This study did not report a significant decrease in prostate cancer risk for individuals administered lycopene, but the study duration of 21 days was likely inadequate to significantly influence prostate cancer risk.

Two other studies examined PSA levels in relation to lycopene administration [12]. One study reported a decline

in PSA in the lycopene as well as placebo group after 1 month of intervention but return to baseline PSA levels for both groups after 4 months of followup [10]. Schwartz et al. [12] did not report a decrease in PSA levels among individuals administered lycopene. In general, the clinical trials have been considerably limited by size, length of study duration, and other methodological issues and do not provide strong support or refutation of an association between lycopene and prostate cancer risk. No adequately sized randomized studies of lycopene for prostate cancer prevention have been conducted.

4.2. Prospective Dietary Studies. Prospective and nested case control studies have been published previously in qualitative [13, 14] and quantitative reviews [15]. In a meta-analysis of prospective studies up to 2003 [15], high intake of raw but not cooked tomatoes was associated with a decreased risk of prostate cancer (relative risk (RR) 0.71, 95% confidence interval (CI): 0.57–0.87). Subsequent cohort studies on dietary lycopene intake [16, 17] have not reported significant inverse associations with prostate cancer risk. However, these studies were conducted in the post-PSA era that likely encompassed a heterogeneous group of prostate cancers that included latent and incident cancers.

Among prospective dietary studies, four [18–21] of six cohorts report an inverse relationship between lycopene or tomato consumption and prostate cancer incidence. The largest and only study with multiple assessments of diet was conducted in the Health Professionals Follow-up Study (HPFS) [18, 19]. The HPFS first reported an inverse association between lycopene intake in 1986 with prostate cancer diagnosed between 1986 and 1992: RR for high versus low quintile of intake = 0.79 (95% CI: 0.64–0.99, $P_{\text{trend}} = 0.04$) [18]. High intake of tomato-based products was associated with a 35% decreased risk of total prostate cancer (RR 0.65, 95% CI: 0.44–0.95) and 53% decreased risk of advanced stage prostate cancer (RR 0.47, 95% CI: 0.22–1.00; $P_{\text{trend}} = 0.03$).

The HPFS analysis was updated for prostate cancer cases between 1992 and 1998 using cumulative average updated intakes (i.e., averaging intake from all the dietary questionnaires up to the time period of risk) of lycopene from 1986 to 1998 with a similar inverse association detected: RR = 0.83 (95% CI: 0.70–0.98, $P_{\text{trend}} = 0.02$) [19]. The HPFS assessed dietary intake every four years, and the timing of intake in relation to period of risk for prostate cancer was assessed. When baseline lycopene intake in 1986 was evaluated for prostate cancer cases during the entire follow-up period, no significant association was seen. However, statistically significant inverse associations were found when using the questionnaire closest in time to the time period of risk (RR for high versus low lycopene intake = 0.84, 95% CI: 0.74–0.96, $P_{\text{trend}} = 0.02$) and cumulative average updated lycopene intake (RR for high versus low lycopene intake = 0.84, 95% CI: 0.73–0.96, $P_{\text{trend}} = 0.003$). These findings suggest that lycopene may be acting relatively late in the carcinogenic process. Alternatively, a single measurement of dietary intake at baseline may not be the best measurement to reflect the potential impact of lycopene in altering prostate carcinogenesis, compared with multiple updated dietary measurements. Individual tomato products were examined in relation to prostate cancer risk, and the strength of the association corresponded to association of the food item with serum lycopene levels, which were concurrently available in the HFPS. For example, tomato sauce, the most bioavailable form of lycopene, was most strongly related to decreased prostate cancer risk (RR for ≥2 servings/week versus <1 serving/month = 0.77, 95% CI: 0.66–0.90), followed by tomato and pizza but not tomato juice. There was an even stronger association for advanced prostate cancer: RR for ≥2 servings/week versus <1 serving/month of tomato sauce = 0.65 (95% CI: 0.42–0.99, $P_{\text{trend}} = 0.02$).

A similar magnitude decrease in prostate cancer was reported in the California Seventh Day Adventist cohort between 1974 and 1982 for men with high tomato consumption [21]. High intake of tomatoes was associated with a statistically significant 40% decreased risk of prostate cancer (RR 0.60, 95% CI: 0.37–0.97). In another study, a 50% lower risk of prostate cancer was reported for high compared with low tomato consumption in US men between 1987 and 1990: RR 0.50 (95% CI: 0.30–0.90, $P_{\text{trend}} = 0.03$), but few details were provided [20].

Dietary cohort studies that did not report associations with prostate cancer incidence frequently had lower lycopene and tomato intake compared with studies that report an association. A dietary cohort study based in The Netherlands between 1986 to 1992 did not report an association between tomato intake and prostate cancer incidence [22]. While this cohort took place between the same time period as the initial HPFS analysis, tomato consumption was low compared with the HPFS. In addition, consumption of tomato-based products, which contain more bioavailable lycopene, was not specifically addressed.

A diet-based cohort study of individuals in the Prostate, Lung, Colorectal, and Ovarian Screening Trial [17] examined intakes of lycopene and top food sources of lycopene but did not find inverse associations for lycopene, raw tomatoes, canned tomatoes, or other processed tomato products (ketchup, tomato sauce, pizza, lasagna, tomato and vegetable juice, chili) with total or nonadvanced prostate cases. Predominately white men (90.7%) enrolled in this study between 1993 and 2001 received baseline PSA screening or digital rectal examination and completed annual questionnaires. Men with a PSA level >4 ng/mL or digital rectal examination concerning for prostate cancer were referred to their medical provider for further diagnostic evaluation and staging of prostate cancer. The majority (92%) of prostate cancers were confirmed for stage and grade. Nonadvanced disease (Gleason score <7 or stage I or II) comprised 61% of total prostate cancer cases and was not associated with total lycopene intake or lycopene from processed foods. Greater consumption of spaghetti/tomato sauce and pizza was associated with decreased incidence of advanced disease but did not reach statistical significance (RR 0.81, 95% CI: 0.57–1.16 and RR 0.79, 95% CI: 0.56–1.10, respectively). The association of tomato products with advanced but not nonadvanced prostate cancer suggests a possible stronger role for lycopene in advanced disease. Limitations of this

study include the assessment of lycopene intake from a single baseline measurement. In addition, intake for all but raw tomatoes was low in this population, with no more than two servings per week (mean total lycopene intake 11,511 and standard deviation 8,498 μg/d). Further, a large portion of prostate cancer cases were diagnosed by initial PSA screening, and total prostate cancers reflected a heterogeneous mix of mostly nonadvanced prostate cancer cases.

Dietary lycopene was not reported to have an association with prostate cancer risk in a prospective study from the Prostate Cancer Prevention Trial [16]. The PCPT originated as a randomized trial of finasteride but was converted to a prospective observational study. Diet was assessed one year after randomization in 1994. Men underwent annual screening for prostate cancer using digital rectal examination and PSA. Men with an abnormal digital rectal examination or PSA level 4 ng/mL or greater were encouraged to receive a prostate biopsy. At the final study year all men not previously diagnosed with prostate cancer were offered prostate biopsies. This resulted in the inclusion of incidental cases of prostate cancer that contributed to the substantial 24.8% of prostate cancer cases diagnosed in men originally randomized to the control group. The majority of cancers were localized and detected by screening or incidentally discovered, and it is possible that low-grade cancers have different characteristics from high-grade cancers. Prior studies of lycopene intake have suggested stronger inverse associations with advanced prostate cancer [18]. In addition, it may be necessary to assess lycopene through simple updated or cumulative average updated intakes rather than a single baseline measurement.

4.3. Prospective Serum and Plasma Studies. A number of studies have examined serum or plasma lycopene from biobanks in relation to subsequent prostate cancer risk. The studies were typically nested case control in design; incident cases and matched cancer-free controls over the follow-up period were identified, and serum or plasma lycopene was measured in the banked biospecimens. In a meta-analysis of prospective studies up to 2003 [15], high serum lycopene levels were associated with significant decreased risk of prostate cancer: RR = 0.78 (95% CI: 0.61–1.00). Subsequent studies on serum lycopene levels conducted in the post-PSA era [23–28] have not reported significant inverse associations with total prostate cancer risk.

One of the earliest studies to report an inverse association between serum lycopene and prostate cancer risk was a nested case control study of 103 prostate cancer cases matched with 103 controls among 25,802 male residents of Washington County, MD who donated blood in 1974 [29]. A nonsignificant 50% reduction in prostate cancer risk was reported (OR 0.50; 95% CI: 0.20–1.29). Two of the largest nested case control studies reported inverse associations between plasma lycopene and prostate cancer risk [30, 31]. Plasma lycopene levels were higher in these multicentered US cohorts compared with other studies, which may reflect the higher education level in these populations. A nested case control study within the Physicians' Health Study, a randomized, placebo-controlled trial of aspirin and β-carotene, assessed incident prostate cancer cases in 578 men compared with 1294 age- and smoking-status-matched controls [30]. Plasma lycopene was lower in cases than controls. Men with higher plasma lycopene had a borderline significant decreased risk of prostate cancer (highest quintile RR = 0.75; 95% CI: 0.54–1.06; $P_{\text{trend}} = 0.05$). There was a significantly greater decreased risk for aggressive (high stage or high grade) prostate cancer: highest quintile plasma lycopene, RR = 0.56 (95% CI: 0.34–0.91; $P_{\text{trend}} = 0.05$). These associations were not confounded by covariates including age, smoking status, body mass index, physical activity, alcohol intake, multivitamin use, or plasma total cholesterol level.

In a nested case control study within the prospective HPFS cohort, 450 incident cases of prostate cancer diagnosed between 1993 and 1998 were matched with 450 controls by age, time, month, season, and year of blood donation [31]. A nonsignificant inverse association was reported for plasma lycopene and risk of prostate cancer: RR for highest versus lowest quintile, 0.66 (95% CI: 0.38–1.13). This association was statistically significant for men older than 65 years at time of plasma donation: RR for highest versus lowest quintile, 0.47 (95% CI: 0.23–0.98), but was not observed for younger men.

Serum lycopene was recently assessed in a nested case control study within the Prostate Cancer Prevention Trial (PCPT) [28]. There was no association between serum lycopene and prostate cancer incidence, but incidentally diagnosed prostate cancer cases by end-of-study biopsies were analyzed alongside prostate cases diagnosed by screening. In a reanalysis that included only cancers diagnosed from abnormal screening there was a significant inverse association between high serum lycopene and prostate cancer [7]. The cancers assessed by end-of-study biopsy were relatively static (e.g., no PSA elevation or sign of clinical progression) during the study period, so may not be appropriately considered as "incident" prostate cancer.

Several other serum lycopene studies reported nonsignificant inverse associations [23–25, 27] or no association [26, 32] with prostate cancer risk. However, these studies were conducted in the post-PSA era that likely encompassed a heterogeneous group of prostate cancers that included latent and incident cancers. As with the PCPT, an association with lycopene could be missed. In the large European study (EPIC) by Key et al., a statistically significant inverse association was observed for cases diagnosed at an advanced stage [26]. In this study, men in the highest versus lowest quintile of lycopene level had a RR of 0.40 (95% CI: 0.19–0.88).

4.4. Case Control Dietary Studies. Several studies retrospectively examined the association between tomatoes, tomato-based products or lycopene, and prostate cancer risk, with mixed results. In a meta-analysis of case control studies through 2003 [15], high intakes of raw tomatoes, cooked tomatoes, and lycopene were not associated with decreased prostate cancer risk.

A strong inverse dose-response relationship between lycopene intake and histopathologically confirmed prostate adenocarcinoma risk was reported in a case control study of Chinese men [33]. Lycopene intake was assessed using a reproducible and validated 130-item food frequency questionnaire for elderly men in China [34]. For lycopene intakes of 1609–3081, 3081–4917, and >4917 μg/d, the RRs of prostate cancer compared with lycopene intake <1609 μg/d were 0.47 (95% CI: 0.25–0.86), 0.40 (95% CI: 0.21–0.77), and 0.17 (95% CI: 0.08–0.39), respectively. The RRs reported for lycopene and prostate cancer in this study were stronger than some prior studies. This study also examined green tea and vegetable and fruit intake and reported strong, significant inverse associations for all these associations. The incidence of prostate cancer is lower in developing countries such as China, compared with Western countries. In China PSA screening is not as commonly widely used compared with Western countries. Prostate cancer is thus usually diagnosed at more advanced stages. While further information and stratification based on prostate cancer grade and staging were not provided in this study, the lower prevalence of PSA screening practices in China compared with the USA suggests prostate cancer cases in this cohort were more likely to be at advanced stages. The strong association between lycopene and probable advanced prostate cancer suggests a role for lycopene in influencing risk of aggressive cancer.

A significant inverse association between tomato intake and prostate cancer was similarly reported in a case control study of 617 Canadian men with prostate cancer and 636 age-matched controls conducted between 1989 and 1993. The RR of prostate cancer was 0.64 (95% CI: 0.45–0.91) for tomato intake >73 g/day compared with <24 g/day. There was no significant association reported for lycopene intake and prostate cancer [35].

In a case control study of 130 prostate cancer cases in Iranian men, tomato consumption of greater than 100 g/week was nonsignificantly inversely associated with decreased prostate cancer risk (RR 0.45; 95% CI: 0.09–2.12) [36]. Food intake was assessed based on the past two months of intake, and tomato intake questions included tomato extract and dressing. However, it is unclear whether this tomato group included both raw and processed tomatoes.

An additional study reported a nonsignificant inverse association between dietary lycopene and prostate cancer risk [37] while several reported no association [38–40].

4.5. Case Control Serum and Plasma Studies. Three case control studies of plasma lycopene reported strong inverse associations with histopathologically confirmed prostate cancer [41–43]. One study of non-Hispanic Caucasian men used high-pressure liquid chromatography (HPLC) to examine plasma lycopene isoforms [42]. An inverse association was reported for *cis*-lycopene-1 only. *cis*-lycopenes 2 through 5 individually and in sum as total *cis*-lycopene and *trans*-lycopene were not associated with prostate cancer risk. This study suggests the structural type of lycopene measured may influence the ability to detect an association, as one cis isomer but not total *cis*- or *trans*-lycopene was associated with decreased prostate cancer risk. Data on individual structural lycopene isomers have been limited. However, a study in the HPFS found that the isomers were highly correlated with each other, making any specific effects difficult to distinguish [44].

The multicentered case control Third National Health and Nutrition Examination Survey (NHANES III) of U.S. Caucasian and African American men aged 40–79 years reported a significant inverse association between serum lycopene and aggressive prostate cancer (highest compared with lowest quartile RR = 0.37; 95% CI: 0.15–0.94; $P_{\text{trend}} = 0.04$) and nonsignificant association between serum lycopene and prostate cancer (highest compared with lowest quartile RR = 0.65; 95% CI: 0.36–1.15; $P_{\text{trend}} = 0.09$) [45]. Caution should be used in interpreting results from case control plasma or serum studies because the cancers could possibly be influencing lycopene level, resulting in reverse causation.

5. Lycopene and Prostate Cancer Associations: Conclusions

This paper focused on prostate cancer incidence that may or may not have been detected by initial PSA screening and highlights differences in the association with lycopene based on prostate cancers initially screened with PSA testing compared with cancers diagnosed in more advanced stages. Elevated PSA may be attributed to a number of benign factors, including the highly prevalent benign prostatic hypertrophy in older men, and should not be used in isolation along with single serum measurements for the diagnosis of prostate cancer. Randomized interventions of lycopene and prostate cancer risk have been limited in scope, and some used PSA as an endpoint [10, 11]. Thus, trials do not provide strong support either for or against a causal association.

The epidemiologic literature on lycopene intake or level and prostate cancer based on observational studies has been inconsistent overall. Earlier studies had appeared more promising but some recent studies are not supportive. There are several potential explanations for this pattern. One potential explanation is that there was a relative over-reporting and publishing of positive studies in the earlier years, followed by a correction of this publication bias as the hypothesis grew in interest and null studies were published. If so, then it may be concluded that there is unlikely to be a causal connection between lycopene intake and risk of prostate cancer.

An alternative possibility is that the earlier studies were conducted largely before the onset of PSA screening, where diagnosis of prostate cancer usually implied a period of increasing aggressive behavior leading to the diagnosis. Thus, the exposure was linked to the development of aggressive behavior in cancers with biologic potential to progress. In the PSA era, 1992–1998 with peak in 1992 following FDA approval as a screening test for prostate cancer, the diagnosis of prostate cancer is not typically linked to aggressive behavior [4, 7]. An example of this phenomenon may be the subgroup of cancers in the PCPT that were diagnosed at end

of study biopsy. There was a high prevalence of undiagnosed prostate cancer, even among the youngest group of men in the study (55–59 years), which suggests that most of the cancers eventually diagnosed during the study period were present at baseline. Throughout the 7-year followup, the cancers diagnosed at end-of-study biopsy showed no evidence of clinical or biochemical progression [28]. Prior studies have shown that most cancers, even many with high-grade Gleason scores, do not progress over prolonged time, and it is well established that only a fraction of prostate cancers result in the most advanced and clinically significant stage and mortality [46]. Thus, the majority of these cancers was likely present at the onset of the study and may be considered static cancers. In fact, when reanalyzed as a case-only study, higher serum lycopene levels in the PCPT appeared to be inversely associated preferentially with cancers that showed evidence of progression relative to cancers that showed no indication of progression. In modeling two patterns of prostate cancer progression, one with low lycopene exposure and rapid tumor growth that reaches a threshold PSA level for clinical diagnosis and the second with high lycopene exposure and slow progression, the latter may be diagnosed at a much later time through an incidental random biopsy rather than PSA. Thus, asymptomatic cancers diagnosed at the end-of-study by biopsy may signify cancers that were inhibited rather than incident cancer. Thus, a possible interpretation is that high levels of lycopene may have inhibited some existing cancers to undergo progression.

Some evidence supports the premise that PSA screening and type of tumor endpoint are critical. For example, the HPFS was analyzed before and after peak PSA testing in the early 1990s. Before PSA testing (1986–1992) tomato sauce intake was inversely associated with prostate cancer incidence and stronger for advanced stage cancers. While the association for total prostate cancer incidence was attenuated during the PSA era (1992–1998), the association with metastatic prostate cancer persisted. In addition, in the large EPIC study [26] serum lycopene level was inversely associated with risk of advanced stage prostate cancer but not nonadvanced prostate cancer.

Additional factors may contribute to the heterogeneity in the literature. As discussed above, studies based on intake are limited by the assessment of intake, food composition databases, and differences in bioavailability. Future studies may be improved by better taking into account bioavailability differences among diverse foods. Prospective studies are preferable to avoid various biases, such as recall bias, or reverse causation in studies of circulating lycopene. With increasing use of PSA, it is becoming increasingly difficult to examine advanced stage prostate cancer, at least in some populations. Examining potential mediators or markers of aggressive behavior in tumor tissue may be another useful approach in the further study of lycopene and prostate cancer risk.

Abbreviations

CI: Confidence interval
HPFS: Health Professionals Follow-up Study
OR: Odds ratio
PCPT: Prostate Cancer Prevention Trial
PSA: Prostate-specific antigen
RR: Relative risk.

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Research Article

Cucurbitacin B Causes Increased Radiation Sensitivity of Human Breast Cancer Cells via G2/M Cell Cycle Arrest

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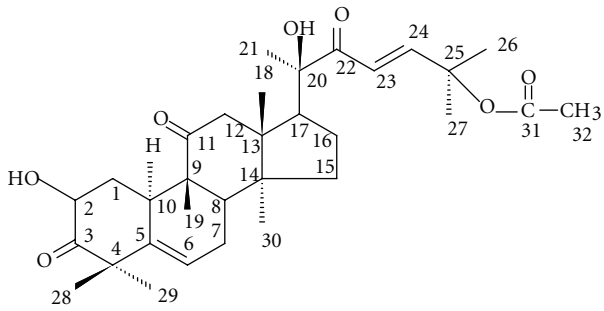
Purpose. To explore the effects of cucurbitacin B on the radiation survival of human breast cancer cells and to elucidate the cellular mechanism of radiosensitization if any. *Materials and Methods*. Human breast carcinoma cell lines were treated with cucurbitacin B before irradiation with 0–10 Gy of ^{137}Cs gamma rays. The effect of cucurbitacin B on cell-survival following irradiation was evaluated by colony-forming assay. Cell cycle distributions were investigated using flow cytometry. Real-time PCR and western blots were performed to investigate the expression of cell cycle checkpoints. *Results*. Cucurbitacin B inhibited breast cancer cell proliferation in a dose-dependent manner. Only MDA-MB-231 and MCF7:5C cells but not SKBR-3 cells were radiosensitized by cucurbitacin B. Flow cytometric analysis for DNA content indicated that cucurbitacin B resulted in G2/M arrest in MDA-MB-231 and MCF7:5C but not SKBR-3 cells. Moreover, Real-time PCR and western blot analysis demonstrated upregulated p21 expression before irradiation, a likely cause of the cell cycle arrest. *Conclusion*. Taken together, these findings suggest that cucurbitacin B causes radiosensitization of some breast cancer cells, and that cucurbitacin B induced G2/M arrest is an important mechanism. Therefore, combinations of cucurbitacin B with radiotherapy may be appropriate for experimental breast cancer treatment.

1. Introduction

Breast cancer is now the most common cause of female cancer and leading cause of cancer deaths among women in the United States and many other parts of the world [1, 2]. Over the past several decades, the incidence of breast cancer has been increasing in economically developed countries [3]. Human epidermal growth factor receptor 2 (Her2) and Estrogen receptor (ER) play critical roles in the development and progression of breast cancer. About 80% of breast cancers are hormone-receptors positive and express estrogen receptors [4]. About 20% of breast cancers do not express estrogen receptor and also Her2 [5, 6]. Therefore, breast cancer that negative for ER and Her2 does not respond to hormonal therapy. Current therapies for the treatment of breast cancer may result in drug resistance or toxicity. There is growing interest in the use of herbs to aid in the maintenance of women's health; it is interesting to use of herbs for the women's health care. Plants contain a wide variety of chemicals that have potent biological effects, including anticancer activity. Natural cucurbitacins are highly oxygenated, tetracyclic triterpenes containing the cucurbitane nucleus skeleton and are predominantly found in plants of the family cucurbitaceae, members of which have long been used in oriental medicines because of the wide range biological activity they exhibited in plants and animals. Among the various cucurbitacins, the most abundant is cucurbitacin B. Cucurbitacin B (Scheme 1) extracted from the Thai herb *Trichosanthes cucumerina* L. has been shown to have anticancer, antimicrobial, and anti-inflammatory activities [7, 8]. Several studies reported that cucurbitacin B and its relatives inhibit the growth of human malignant cells both *in vitro* and *in vivo* including breast cancer [9], head and neck squamous cell carcinoma [10], pancreatic



Cucurbitacin B ($C_{32}H_{46}O_8$)
25-acetoxy-2β, 16α, 20β-trihydroxy-9β
-methyl-19nor-10α-lanosta-5, 23-diene3, 11, 22-trione

Scheme 1: Structure of cucurbitacin B.

cancer [11], hepatocellular carcinoma [12], osteosarcoma [13], and myeloid leukemia [14]. Our previous report has shown that cucurbitacin B exerts anticancer effect by inhibiting telomerase via downregulating both the *hTERT* and *c-Myc* expression and arrest of the cell cycle at G_2/M phase in breast cancer cells [15]. Some studies have reported that cells are most sensitive to radiation in G_2/M and most resistant in S phase [16]. For example, synchronized Chinese hamster cells were most sensitive to irradiation during mitosis and in G_2 phase and less sensitive in G_1 and the latter past of S phase [17, 18]. Drugs, including Docetaxel, that arrest cells cell in G_2/M phase of cell cycle have been demonstrated as radiosensitizer agent [19]. The aims of this study were to determine the radiosensitizing potential of cucurbitacin B in human breast cancer cells and to elucidate the cellular mechanism of the radiosensitization. In the present study, we demonstrated that cucurbitacin B sensitizes human breast cancer cells to radiation by inducing them to accumulate in G_2/M phase of the cell cycle.

2. Materials and Methods

2.1. Cell Lines and Drug Treatment. Human breast cancer cell lines (SKBR−3 (ER−/Her2+), MDA-MB-231 (ER−/Her2−), and hormone-independent MCF7:5C (ER−/Her2−)) was cultured at 37°C under a 5% CO_2 atmosphere. SKBR-3 breast cancer cells were maintained in McCoy's 5A medium. MDA-MB-231 and MCF7:5C were maintained in DMEM/F12 medium. All medium were supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin.

2.2. Drug and Radiation Treatment. Cucurbitacin B was authenticated by Professor Dr. Apichart suksamrarn from Faculty of Science, Ramkhamhaeng University, Bangkok, Thailand. This compound was dissolved in 10% dimethylsulfoxide (DMSO) and diluted with DMEM/F12 medium and McCoy's 5A medium to the desired concentrations prior to use. A cesium machine was used to radiate cells with a dose ranging from 0 to 10 Gy.

2.3. Clonogenic Survival Assay. Cells were seeded in a 100-mm culture plate and treated with the indicated concentration of cucurbitacin B for 48 hr prior to radiation exposure. After exposure, cells were then trypsinized and seed on the basis of difference density in a 60 mm culture plate with 5 mL of medium. The plates were incubated at 37°C under a 5% CO_2 atmosphere for 14–21 day. The cells were fixed in ethanol and stained with crystal violet. Colonies containing more than 50 cells were counted as survivors. Surviving fractions were calculated by normalization to the plating efficiency of appropriate control groups.

2.4. Cell Cycle Analysis. For cell cycle analysis, cells were treated with cucurbitacin B at various concentrations for 48 hr and harvested. The cells were trypsinized and resuspend in 1 mL DPBS. One million cells were centrifuged and suspended in 0.5 mL of Krishan reagent (0.1% Na citrate, 0.03% NP-40, 0.05 mg/mL PI, 0.02 mg/mL RNase A) before analysis. The stained cells were subjected to DNA content/cell cycle analysis using an LSR flow cytometer.

2.5. Apoptosis Analysis. For apoptosis, the Annexin V-FITC Apoptosis Detection Kit (BD bioscience, Bedford, MA) was used to assess annexin V-positive cells. Briefly, fresh cell preparations were incubated with 1x annexin binding buffer and annexin V-FITC- (2.5 μg/mL) conjugated primary antibody for 15 min on ice. After incubation, propidium iodide (PI; 10 μg/mL) was added to the suspension and the cells were analyzed by flow cytometry using an LSR flow cytometer.

2.6. Quantification of p21 mRNA. Cells (5×10^5 cells/well) were seeded into 6-well plate and treated with various concentration of cucurbitacin B for 48 hr. Total RNA was isolated from each cell line using the Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA). Two micrograms of total RNA were reverse-transcribed with random primer according to the manufacture's protocol using High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA). Real-time PCR was performed using Fast SYBR Green Master Mix (Applied Biosystems) with the Applied Biosystems 7500 Fast Real-Time PCR system (Applied Biosystems). The PCR primers set were as follows: sense5′-TGAGCCGCGACTGTGATG-3′ and anti-sense5′-GTCTCGGTGACAAAGTCGAAGTT-3′ for *p21 and* sense5′-GAAGGTGAAGGTCGGAGTC-3′ and anti-sense5′-GAAGATGGTGATGGGATTTC-3′ for *GAPDH*. The relative ratio of *p21* was then calculated using the formula: $2^{-\Delta\Delta Ct} = 2^{-\{\Delta Ct(\text{Cucurbitacin B-treated}) - \Delta Ct(\text{untreated})\}}$, where $\Delta Ct = Ct(p21) - Ct(GAPDH)$.

2.7. Western Blot Analysis. After cucurbitacin B treatment, cell pellets were collected and lysed with 100 μL RIPA cell-lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 0.1% SDS, 0.5% Na Deoxycholate, 1% TX-100) plus 1 mM NaF, 10 mM $NaVO_4$, 10 mM PMSF, and 1/100 protease inhibitor cocktail (Sigma). Total protein was determined using Bio-Rad protein assay (Life science, Hercules, CA). Equal amount

of proteins were separated by 12.5% SDS-Polyacrylamide gels and electrotransferred onto nitrocellulose membranes and treated with anti p21 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) overnight. Equal protein loading was confirmed on all immunoblots using beta-tubulin antibody (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA) at a dilution 500. Goat anti-rabbit IgG (BD Transduction Laboratories, San Diego, CA) was used as a secondary antibody against all primary antibodies. Bands were visualized by chemiluminescence with ECL plus reagent (Pierce, Rockford, IL) on a Typhoon FLA 7000.

2.8. Statistical Analysis. All experiments were performed at least three times. Statistical analysis was performed using one-way ANOVA to compare the effect among control (without cucurbitacin) and treated cells. P value < 0.05 was considered statistically significant.

3. Results

3.1. Cucurbitacin B Induced Clonogenic Inhibition of Breast Cancer Cells. The inhibitory effect of cucurbitacin B on colony formation in human breast cancer cells was evaluated by clonogenic assay. Cells were incubated with cucurbitacin B alone for 48 hr and then allowed to form colonies in fresh medium. The surviving fraction as a function of drug concentration is shown in Figure 1. The average 50% (IC_{50}) inhibitory concentrations for clonogenic cell death in three cells was 3.2, 2.4, and 1.9 μM for MCF7:5C, and MDA-MB-231, and SKBR-3 respectively. The results are the average from three independent experiments for each cell lines. In the clonogenic assay SKBR-3, was the most sensitive cell to cucurbitacin B under the same condition for other cells.

3.2. Cell Cycle. Effect of cucurbitacin B on cell cycle progression in MCF7:5C, MDA-MB-231, and SKBR-3 cells were analyzed according to the principle of the DNA content in each phase of, cell cycle. Cells were treated with cucurbitacin B for 48 hr, and DNA content was analyzed via flow cytometry. MCF7:5C and MDA-MB-231 cells after treated were arrested at G_2/M phase of cell cycle with a decrease of cells population in G1 and S phase of cell cycle, as was observed in several cancer cell lines. However, in contrast to the effect of cucurbitacin B on MCF7:5C and MDA-MB-231 cells, cucurbitacin B did not contribute to G_2/M phase arrest in SKBR-3 cells (Figure 2).

3.3. Apoptosis Effect of Cucurbitacin B on Breast Cancer Cells. Apoptosis effect of cucurbitacin B was evaluated by using Annexin V-FITC and propidium iodide staining. This assay revealed that the negatively charged phospholipids phosphatidylserine found on the interior surface of the plasma membrane of the cells is trans-located to the cell surface during apoptosis. After 48 hr incubation with 0 μM, 2.5 μM, and 5 μM of cucurbitacin B, cells were stained and subjected to bivariate flow cytometric analysis. As shown in Figure 3, untreated cells did not show any significant

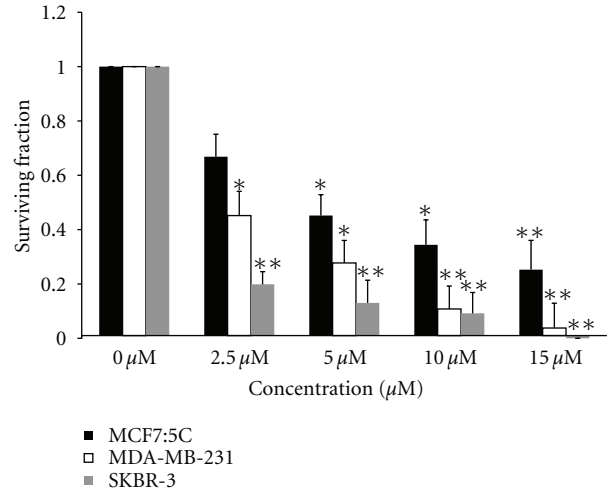


Figure 1: The inhibitory effects of cucurbitacin B on colony formation in breast cancer cells. MCF7:5C, MDA-MB-231, and SKBR-3 were treated with the indicated concentration of cucurbitacin B for 48 hr. After incubating, cells were seeded on the basis of difference density in a 60 mm culture plate with 5 mL of fresh medium. At 14 days after seeding, colonies were fixed and stained with 0.1% crystal violet. Results shown are the average of three independent experiments. $^{*}P < 0.05$ versus nontreated control, $^{**}P < 0.01$ versus nontreated control.

apoptosis, whereas cells become apoptotic with cucurbitacin B treatment at the indicated concentration in all cells.

3.3.1. p21 mRNA Expression. To determine the effect of cucurbitacin B on *p21* mRNA expression, all three cell lines were incubated with cucurbitacin B for 48 hr, in parallel with untreated cell. Exposure of MCF7:5C, MDA-MB-231, and SKBR-3 cell lines to 2.5 μM and 5 μM of cucurbitacin B resulted in the progressive increase *p21* mRNA level. SKBR-3 shows the highest induction of p21 mRNA expression after cucurbitacin B treatment. The expression *p21* mRNA in SKBR-3 was increased up to 20 times when compared with untreated cell while MCF7:5C and MDA-MB-231 was increased 3-4 times as shown in Figure 4(a). The real-time PCR products were applied on 0.8% agarose gel containing ethidium bromide (EtBr) to scrutinize that the PCR reaction was specific and that cucurbitacin B induced gene expression of *p21*.

3.3.2. Upregulation of p21 Protein by Cucurbitacin B. We examined the effect of cucurbitacin B on the expression of cell cycle regulated protein by western blot analysis. Cells were incubated with the indicated concentration of cucurbitacin B for 48 hr and total protein were extracted for western blot analysis. As shown in Figure 5, protein expression of cyclin-dependent kinase inhibitor p21 was significantly increased following cucurbitacin B treatment in all study cells. SKBR-3 cells, which showed the highest mRNA accumulation in response to cucurbitacin B, also showed the greatest induction of the p21 protein; however, this was not

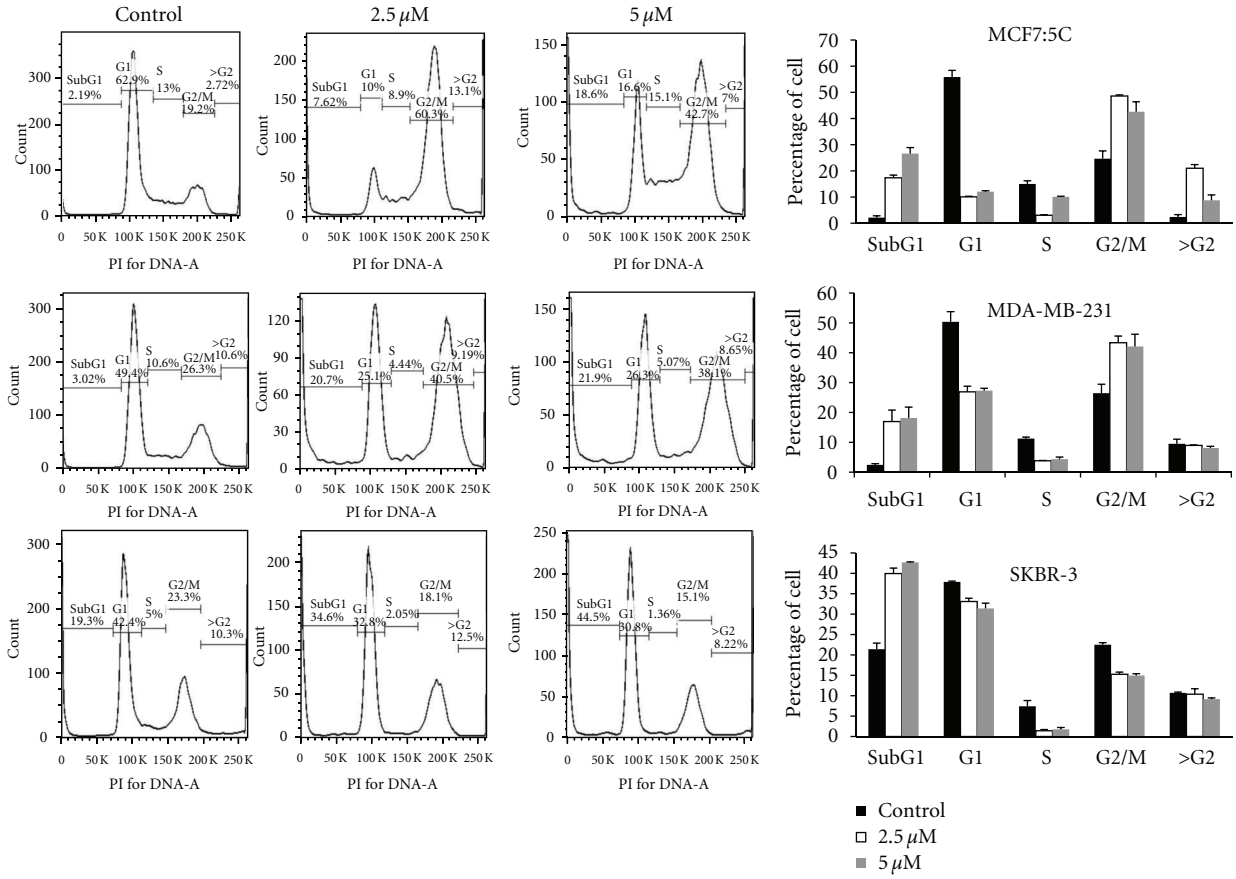


Figure 2: Effect of cucurbitacin B on the cell cycle progression of breast cancer cells. MCF7:5C, MDA-MB-231, and SKBR-3 were treated with cucurbitacin B for 48 hr, and then stained with propidium iodide (PI) and subjected to flow cytometric analysis. The DNA histograms shown are representative of three independent experiments. Blockage at G_2/M and apoptotic induction was observed ($SubG_1$).

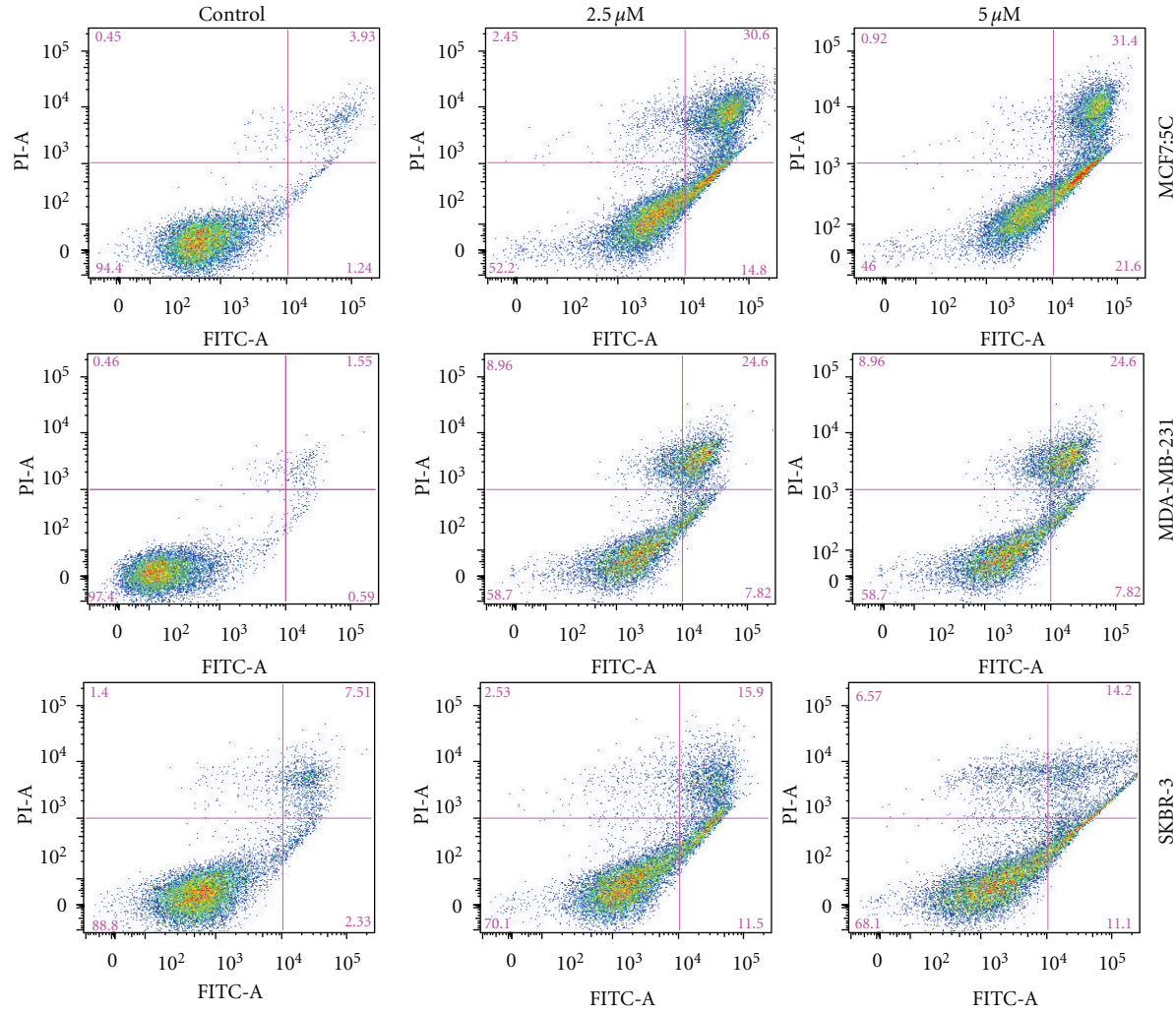
necessarily linked to G_2/M arrest or radiation sensitization by cucurbitacin B.

3.4. The Radiopotentiating Effect of CuB on Breast Cancer Cells. To determine whether cucurbitacin B sensitized human breast cancer cells to ionizing radiation, all three cell lines were treated with 5 μM cucurbitacin B for 48 hr following irradiation with a ^{137}Cs gamma-irradiator at doses ranging 0–8 Gy. The cells were then allowed to form colonies in fresh medium. The plating efficiency of all cells was between 60 and 80%. Figure 6 shows the radiation survival curves derived from clonogenic assays of the three cell lines irradiated after 48 hr incubation with cucurbitacin B. The latter slope of survival curve of MCF7:C and MDA-MB-231 cells for radiation and cucurbitacin B treated were greater than radiation only, especially at the 6 and 8 Gy radiation doses, indicating that cucurbitacin B treatment augmented the effects of radiation in both cell lines where G_2/M arrest was observed to occur. However, SKBR-3 did not show to augment the effects of radiation, consistent with the cell cycle distribution shown in Figure 2 and the absence of a G_2/M arrest in SKBR-3 cells.

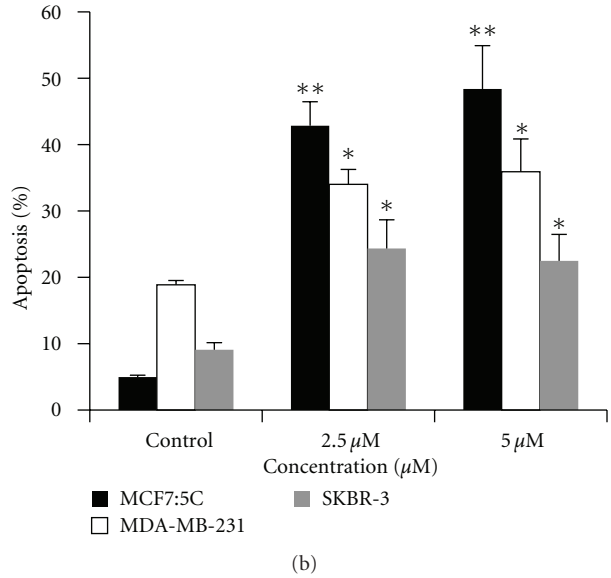
4. Discussion

The Cucurbitacins are highly oxygenated, tetracyclic triterpenes containing the cucurbitane nucleus skeleton and are of great interest because of the wide range biological activity they exhibited in plants and animals. Cucurbitacins have been reported to inhibit several types of cancers. Cucurbitacins are divided into twelve categories [20]. Among the various cucurbitacins, the most abundant is cucurbitacin B [21]. Many reports have shown that cucurbitacin B has potent antiproliferative effect on breast cancer cells. For instance, cucurbitacin B, extracted from root and fruit juice of *T. cucumerina* has been reported to exert the cytotoxicity on human breast cancer cell lines [22]. Wakimoto et al. [9] reported that cucurbitacin B exerts the anticancer activity against ER−, Her2/neu amplified, and p53 mutant breast cancers both *in vitro* and *in vivo* [9]. Similarly, cucurbitacin B has been reported to inhibit Wnt signaling pathway through reduction of Wnt-associated protein and reduced translocation of galectin-3-mediated β-catenin to the nucleus [23].

In this study, we analyzed the anticancer activity of cucurbitacin B in human breast cancer cells lines: MCF7:5C, MDA-MB-231, and SKBR-3 using clonogenic survival assay. Among these three cells lines, SKBR-3 express high levels of



(a)



(b)

Figure 3: Cell death of breast cancer cells induced by cucurbitacin B. MCF7:5C, MDA-MB-231, and SKBR-3 were incubated with cucurbitacin B for 48 hr and apoptosis was analyzed by staining phosphatidylserine translocation with FITC-Annexin V. Annexin V staining is represented on the x-axis and PI staining is represented on the y-axis (a). The most representative result of three independent experiments is shown. Simple vertical bars represent the mean apoptosis rate of all of breast cancer cells (b). Results shown are the average of three independent experiments. $^{*}P < 0.05$ versus nontreated control, $^{**}P < 0.01$ versus nontreated control.

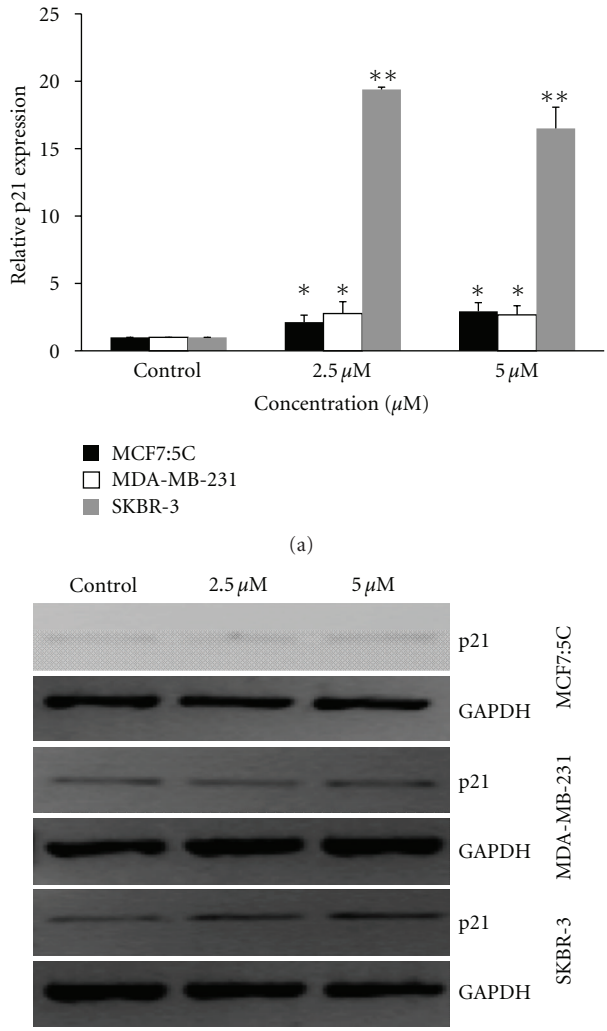


Figure 4: Effect of cucurbitacin B on *p21* gene expression. MCF7:5C, MDA-MB-231, and SKBR-3 were incubated for 48 hr with the specified concentrations of cucurbitacin B, and RNA was extracted for real-time PCR to quantitate the expression level of *p21*. Relative expression levels of *p21* mRNA at indicated concentration. Results shown are the average of three independent experiments. $^{*}P < 0.05$ versus nontreated control, $^{**}P < 0.01$ versus nontreated control.

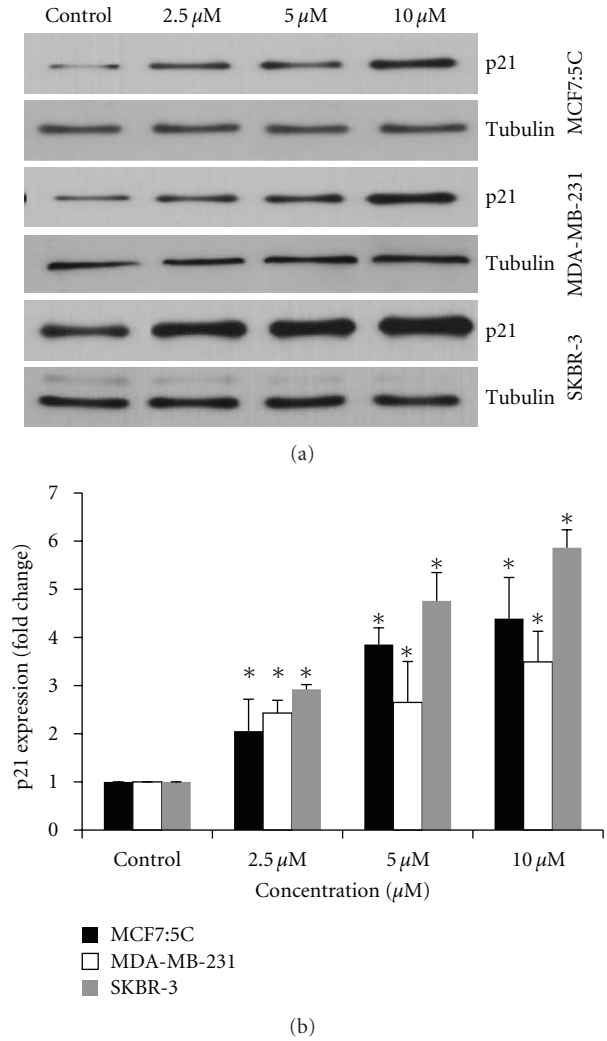


Figure 5: Effect of cucurbitacin B on p21 protein expression in breast cancer cells. (a) Cells were treated with cucurbitacin B for 48 hr, and then the total proteins were extracted and performed western blotting to analyze *p*21 expression. Tubulin was used as an equal loading control. (b) Densitometric analyses of expression of *p*21 relative to the untreated control. $^{*}P < 0.05$ versus nontreated control, $^{**}P < 0.01$ versus nontreated control.

Her2/neu receptor, whereas MCF7:5C and MDA-MB-231 do not express these receptor. We showed that cucurbitacin B has potent antiproliferation activity in all cell types. SKBR-3 is the most sensitive to cucurbitacin B when compared with other two cell lines. We further determined the effect of cucurbitacin B on cell cycle progression and apoptosis induction in breast cancer cell lines. The results showed that apoptotic cells were induced by cucurbitacin B treatment in all cell lines, and cell cycle is arrested at the G_2/M phase in MCF7:5C and MDA-MB-231 cells but not in SKBR-3 cells (Figure 2). Several authors have shown that the radiosensitizing effect of anticancer drug is due to cell cycle alteration. Paclitaxel, a microtubule-stabilizing drug, has been shown to enhance radiosensitivity by blocking cells in G_2/M phase of the cell cycle [24, 25]. Since cells in the G_2/M phase have been reported to be more radiosensitive than in other phases of cell cycle, and cucurbitacin B treatment increases the number of cells in G_2/M phase of the cell cycle and thus enhanced the effects of radiation on breast cancer cell line. In our study, cucurbitacin B exerted the radiosensitivity when administered 5 μM of cucurbitacin B before radiation on MCF7:5C and MDA-MB-231 cells. However, no radiosensitization occurred when SKBR-3 cells were exposed to cucurbitacin B at 5 μM and radiation, and no accumulation of cells in the G_2/M phase was observed prior to the start of irradiation under these conditions. p21 and cell cycle checkpoints were shown to modulate the nucleotide excision repair process to facilitate the repair of

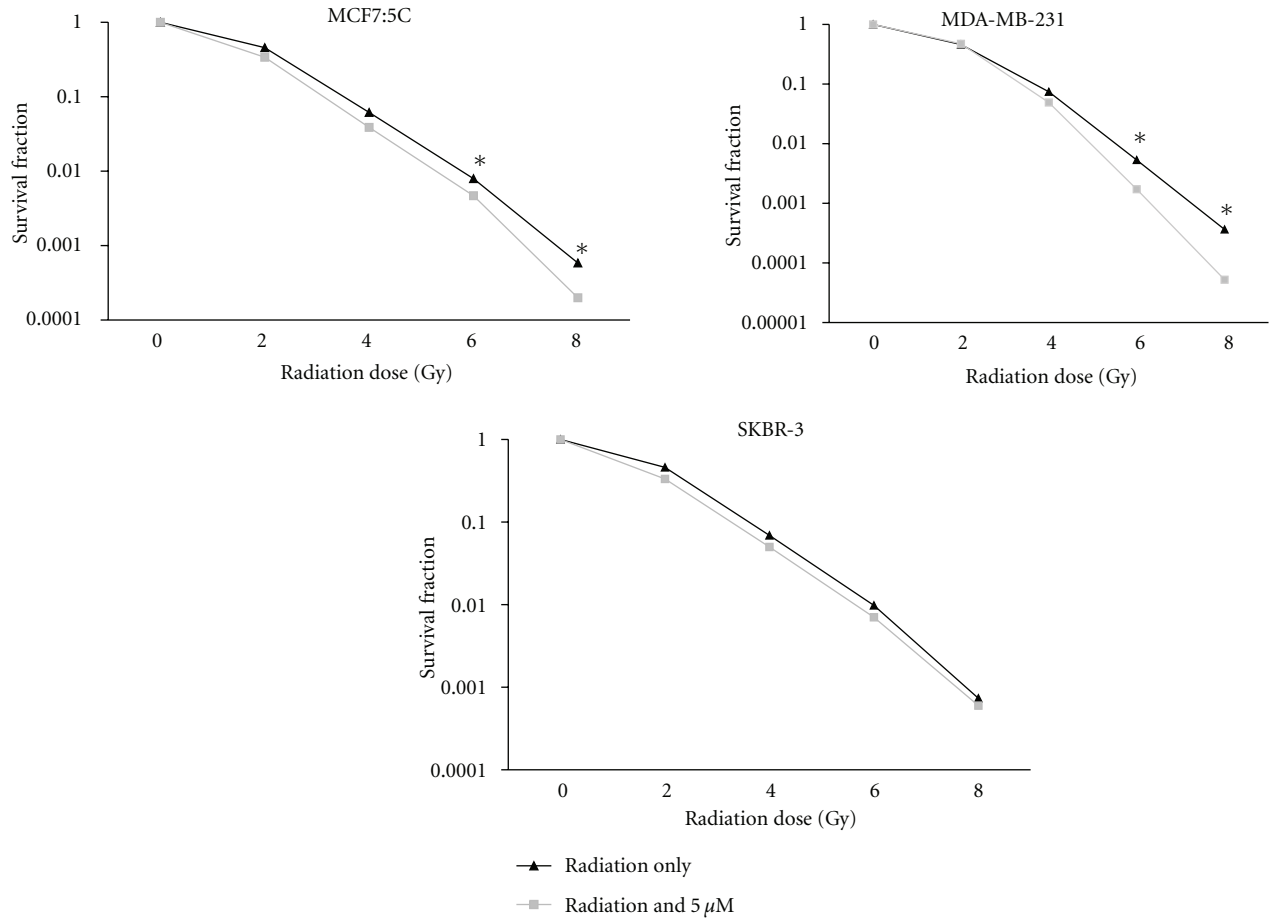


Figure 6: Clonogenic survival of breast cancer cells after treatment including irradiation with or without cucurbitacin B. MCF7:5C, MDA-MB-231, and SKBR-3 cells were treated with 5 μM of cucurbitacin B for 48 hr before radiation. Following the incubation period with specific drug concentration, cells were harvested, resuspended in fresh medium, and then irradiated at 0–8 Gy. Colony formation was detected by 21 days later and survival curve was constructed. Data was a summary of three experiments.

DNA damage even in the absence of wild-type p53 [26]. Our results indicate that p21 expression level by cucurbitacin B treatment mostly showed upregulation in all cell lines especially in SKBR-3 which showed the highest induction when compared with other cell types (Figures 4 and 5). Therefore, no radiosensitization was manifest using 5 μM cucurbitacin B in SKBR-3 likely because cucurbitacin B did not induce a G_2/M cell cycle arrest. Taken together, the radiosensitizing effect by cucurbitacin B was dependent on the induction of G_2/M arrest in breast cancer cells but not necessarily on the induction of p21. In summary, cucurbitacin B can enhance the effect of radiosensitization on breast cancer cells and studies *in vivo* are required to evaluate the biological efficacy of cucurbitacin B treatment.

Conflict of Interests

The authors report no conflict of interests. The authors alone are responsible for the content and writing of the paper.

Acknowledgments

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Research Article

Synergistic Effect of Garcinol and Curcumin on Antiproliferative and Apoptotic Activity in Pancreatic Cancer Cells

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Pancreatic cancer (PaCa) is a major health concern due to its aggressiveness and early metastasis. Current treatments for PaCa are limited by development of resistance against therapy. As an alternative strategy, we assessed the combinatorial effect of dietary compounds, garcinol and curcumin, on human PaCa cells (BxPC-3 and Panc-1). A significant ($P < 0.05$) dose-dependent reduction in cell viability and increase in apoptosis were observed in both cell lines as compared to untreated controls. A combination index (CI) value < 1, for a two-way comparison of curcumin and garcinol, suggests synergism. The potency (*Dm*) of the combination of garcinol and curcumin was 2 to 10 fold that of the individual agents. This indicates that curcumin and garcinol in combination exhibit a high level of synergism, with enhanced bioactivity, thereby reducing the required effective dose required for each individually. This combinatorial strategy may hold promise in future development of therapies against PaCa.

1. Introduction

Cancer is a major health concern across the globe today with pancreatic cancer (PaCa) being the fifth major cause of deaths due to cancer in the United States. Development and progression of this chronic disease involves deregulation and activation of multiple signaling pathways at different stages of carcinogenesis. This complexity associated with the disease causes limitations in designing high-efficacy therapeutic strategies. Although immense work has been done in the prevention and treatment of PaCa, the results are not satisfactory and need improvement. For example, gemcitabine is a standard cytotoxic chemotherapeutic agent used for treatment in PaCa. However, this drug provides limited survival advantage along with several side effects and development of chemoresistance [1, 2]. Therefore, exploring new approaches for better maintenance of this disease is of great interest.

Epidemiological studies have consistently shown that consumption of a healthy diet including fruits, vegetables, and whole grains is strongly associated with reduced risk of cancer and other diseases [3]. Over the years, several natural bioactive compounds or phytochemicals have been isolated, identified, and their potential anticarcinogenic properties have been evaluated. However, efficacy of interactions amongst various dietary components needs further analysis. There is a growing body of evidence that chemotherapeutic combination strategies would be more efficient in reducing drug toxicity, inhibiting tumor development and progression than with either agent alone. Research suggests that increased consumption of curcumin isolated from *Curcuma longa* or Turmeric, contributes to lower risk of cancer in Asian (Indian) populations as compared to industrialized western nations [4, 5]. Also recently, the anticancer properties of garcinol, a bioactive compound derived from the rind of the fruit, *Garcinia indica,* Kokum or Mangosteen has been revealed. The anticarcinogenic properties of this compound in several cancers such as breast, oral, and colon cancer have been tested. Very recently, we and others have showed that garcinol reduces pancreatic cancer cell viability and increases apoptosis via downregulation of nuclear factor NF-κB [6, 7]. Asian (Indian) populations consume turmeric and kokum as a spice in their diet regularly. It is possible that the lower incidence of pancreatic cancer in India (according to 2008 statistics, 2 cases per 100,000 persons per year) as compared

to the western nations (in 2008, an estimated 37,680 new cases of pancreatic cancer (18,770 in men and 18,910 in women) were diagnosed in the United States) [3, 8] might be attributed in part from interactions between the bioactive components in diet including that of turmeric and kokum.

When a combination of two or more compounds exhibit a more potent therapeutic effect than that of individual compounds at equal concentrations, the effect is described to be a synergistic one. In this study, we tested the hypothesis that the bioactive compounds garcinol from *Garcinia indica* and curcumin derived from *Curcuma longa* will work in synergism and inhibit the growth of PaCa cells. Therefore, garcinol, curcumin, and their combination were tested in PaCa cell lines BxPC-3 and Panc-1, carrying wildtype and mutated *K-ras,* respectively. Our results indicate that the combination of garcinol and curcumin significantly inhibited cell viability ($P < 0.05$) and caused induction of apoptosis via upregulation of caspase-3 and -9 activity ($P < 0.05$) in both cell lines. The combination proved to be synergistic and/or additive. These data suggest a potential for the combination therapy for improvement of efficacy in PaCa treatment and hence warrants further investigation.

2. Material and Methods

2.1. Cell Culture. Human pancreatic carcinoma cell lines BxPC-3 and Panc-1 were obtained from American Type Culture Collection (Manassas, VA, USA). The cell lines were maintained in continuous exponential growth by twice a week passage in RPMI-1640 medium (Cellgro Manassas, VA; BxPC-3 cells) and Dulbecco modified Eagle's medium (*DM*EM, Cellgro Manassas, VA; Panc-1 cells), respectively and supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 10 mg/mL streptomycin in a humidified incubator containing 5% CO_2 in air at 37°C. Each cell line was split regularly before attaining 70–80% confluence.

2.2. Reagents. Garcinol (≥95% (TLC), Biomol International, USA) and curcumin (≥94% (curcuminoid content), ≥80% (Curcumin) Sigma Aldrich, USA) were dissolved in *DM*SO to make 20 mM stock solution.

2.3. Cell Viability. Cells were seeded at a density of 3×10^3 cells/well in 96-well microtiter culture plates. After overnight incubation, the medium was removed and replaced with fresh medium containing different concentrations of garcinol (0–40 μM) diluted from a 20 mM stock and/or curcumin (0–50 μM). After 72 hrs, MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) solution was added to each well and incubated further for one hour. Color development was measured spectrophotometrically at 595 nm on a plate reader (BIO-TEK Instruments) and quantified as per the manufacturer's protocol (Promega, USA). Cell viability has been expressed as a percentage, for each treatment group relative to control in the absence of garcinol or curcumin.

2.4. Quantification of Apoptosis. The cell apoptosis ELISA Detection Kit (Roche, Palo Alto, CA, USA) was used to detect apoptosis according to the manufacturer's protocol. Briefly, after treatment of BxPC-3 and Panc-1 cells with garcinol or curcumin for 48 hrs, the cytoplasmic histone/DNA fragments from cells were extracted and bound to immobilized antihistone antibody. Subsequently, a peroxidase-conjugated anti-DNA antibody was used for the detection of immobilized histone/DNA fragments. After addition of the peroxidase substrate, the absorbance by the samples was determined at 405 nm with an ULTRA Multifunctional Microplate Reader (BIO-TEK Instruments).

2.5. Morphological Changes. Morphological changes characteristic of apoptosis were determined by DAPI (4′, 6-diamidino-2-phenylindole) staining as per manufacturer's protocol (Invitrogen, USA). Briefly, 5×10^3 cells were seeded into 6-well plates containing 1-2 mL medium. After 24–36 hrs, garcinol and/or curcumin was added and incubated for another 48 hrs. Cells were harvested by trypsinization, washed with PBS, and subsequently incubated for 30 minutes with DAPI at room temperature in dark for 30 minutes. Prior to microscopic analysis, the cells were stained with Prolong Gold Antifade reagent and visualized under Fluorescence Microscope (Nikon Eclipse, 80i) with an excitation maximum at 358 nm and an emission maximum at 461 nm.

2.6. Caspase Activity. Caspase-3 and -9 activities were measured in whole-cell lysates prepared from garcinol and/or curcumin-treated samples using a commercially available assay kit (R&D Assay System, Minneapolis, MN, USA) according to the manufacturer's instructions.

2.7. Analysis of Cytotoxic Synergy. Cells were plated as described above and allowed to attach overnight. The culture medium was replaced with fresh medium containing curcumin and garcinol individually or in combination in different ratios of 1 : 2.5, 1 : 5, and 1 : 10 for 72 hrs, and the effect on cell growth was examined by the MTS assay method as described above and then analyzed using the Calcusyn (Biosoft) software program, which utilizes the T.C. Chou method of determining synergy and antagonism.

The combination index was determined at a 25%, 50%, and 75% toxicity level for each cell line and at each drug ratio. The median-effect equation and combination index (CI) analysis was used to calculate the interaction between treatment modalities. This analysis determines if the effect of the combination is antagonistic, additive, or synergistic. A CI value of one indicates that the effect of one drug is additive to the second, a CI value of greater than one indicates antagonism between the two agents, and a CI value of less than one indicates synergism between the agents. The equation used to calculate CI is as follows: $(D)1/(Dx)1 + (D)2/(Dx)2 + \alpha(D)1(D)2/(Dx)1(Dx)2$, where $(Dx)1$ and $(Dx)2$ are the doses for x% inhibition by drug 1 and drug 2 alone [9]. These values are obtained from the median-effect equation. $(D)1$ and $(D)2$ are the doses in combination that inhibit cell growth by x%. A more detailed description of degrees of synergism and antagonism is adapted from Chou and Hayball (CalcuSyn, Windows software for dose effect analysis. Cambridge: Biosoft, 1996).

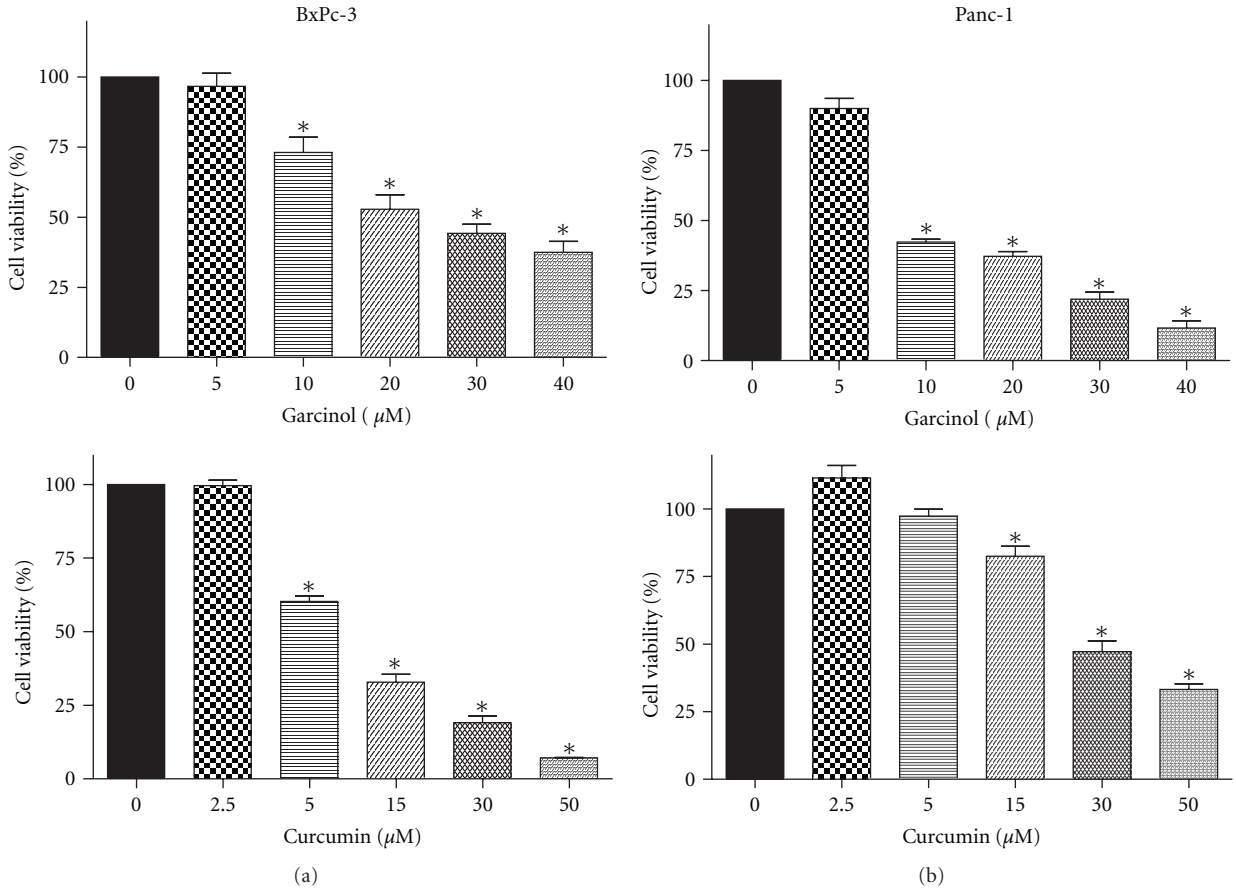

Figure 1: Percentage of metabolically viable cells was reduced in a dose-responsive manner on 48 hr treatment with garcinol (upper panel) or curcumin (lower panel) in both PaCa cell lines. (a) BxPC-3 and (b) Panc-1 as analyzed using MTS assay. $^*P < 0.05$ relative to control.

3. Results and Discussion

3.1. Cell Viability. BxPC-3 and Panc-1 cells were tested for their effects on cell viability under the influence of curcumin or garcinol. Both cell lines are p53 mutated with differences in their *K-ras* mutation status. Panc-1 is a poorly differentiated PaCa cell line with a mutated *K-ras,* whereas BxPC-3 is a moderately differentiated PaCa cell line with a wild type *K-ras*. We observed that both curcumin and garcinol significantly ($P < 0.05$) reduced cell viability in both cell lines in a dose-dependent manner. However, garcinol exhibited a more potent effect (IC50 = ~7 μM) in Panc-1 cells which was comparable to the efficacy of curcumin (IC50 = ~10 μM) in BxPC-3 cells as seen in Figure 1. Cells with mutant oncogenes such as *K-ras* continue to grow even when they are not receiving any growth signals. Both the agents reduced cell viability but to varying extents.

Garcinol and curcumin hold structural resemblance, but our results suggest that their therapeutic mechanistic targets might be different. Overall, this indicates that garcinol might play an important role in targeting the *K-ras* pathway. In BxPC-3 cells (Figure 1(a)) IC50 for garcinol treatment (upper panel) was observed at approximately 15 μM as compared to 10 μM curcumin treatment for 48 hours. In addition, in Panc-1 cells (Figure 1(b)) IC50 values for garcinol (upper panel) and curcumin treatment were 7 μM and 25 μM, respectively. We and others have consistently reported no toxicity at treatment dose of either curcumin [10] or garcinol [6, 7] on normal cells. Hence, these two agents in combination could be a useful strategy for the treatment of this aggressive disease.

3.2. Apoptotic Induction. In order to confirm whether the reduction in cell viability was in part due to apoptotic induction, we performed the ELISA assays and saw that both garcinol (upper panel) and curcumin (lower panel) induced apoptosis in a dose-dependent manner in both PaCa cell lines, BxPC-3 (Figure 2(a)) and Panc-1 (Figure 2(b)). Normal cells become malignant when cellular tumor suppressor genes are rendered nonfunctional through mutations. Both Panc-1 and BxPC-3 exhibit a p53 mutation. p53 is a key tumor suppressor protein that limits cell proliferation by inducing cell cycle arrest and apoptosis in response to cellular stress. Hence, the role of p53-independent pathways such as retinoblastoma protein (Rb) or p73α in induction of apoptosis needs further investigation. We determined morphological changes associated with apoptosis, such as formation of apoptotic bodies and reduction in cell number using the DAPI stain as in Figures 2(c) and 2(d) in both PaCa cell lines upon treatment.

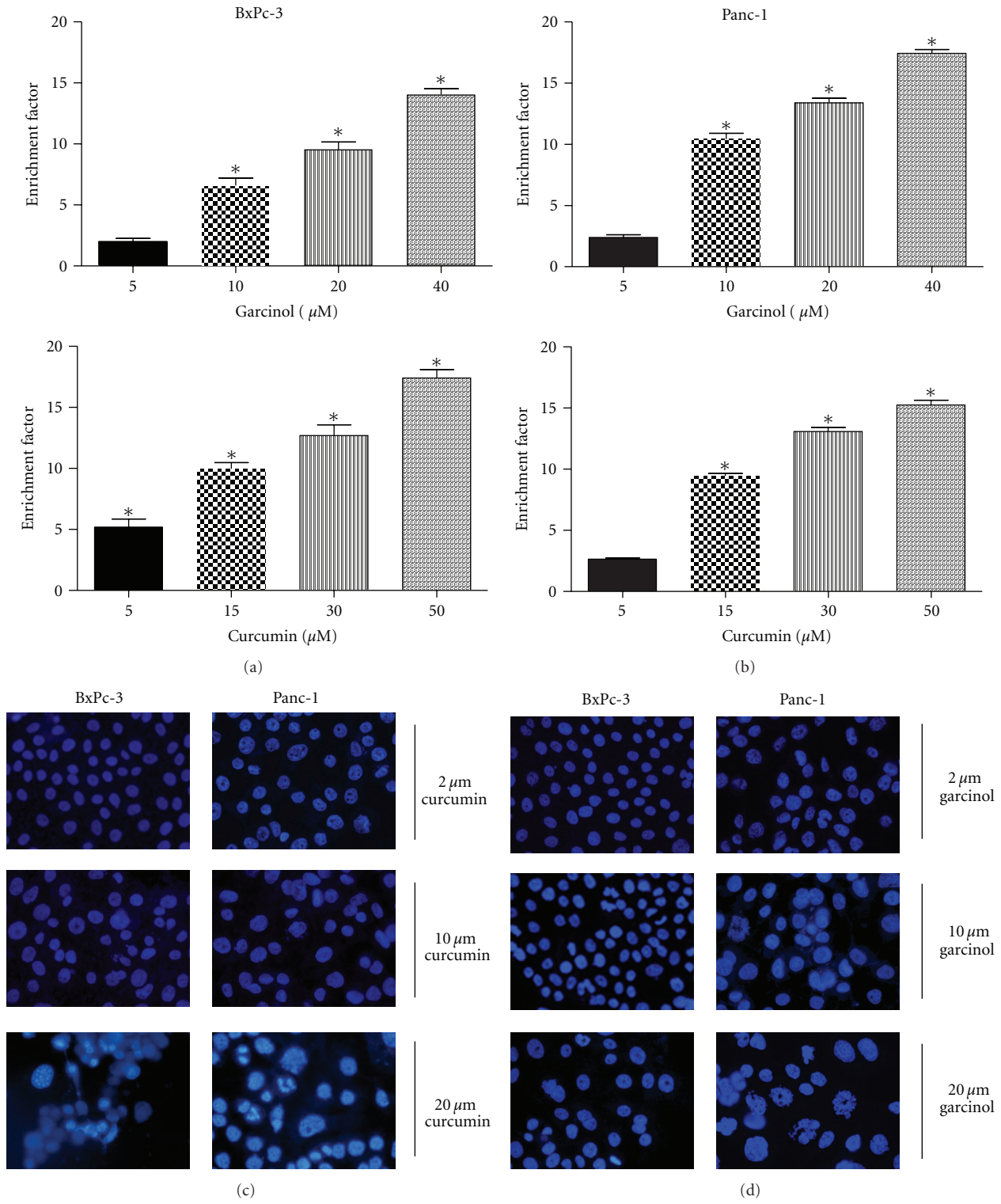

Figure 2: Garcinol-(upper panel) or curcumin-(lower panel) treated cytosolic extracts were used to evaluate induction of apoptosis in PaCa cells. (a) BxPC-3 and (b) Panc-1 using ELISA-Histone DNA Enrichment Assay. Results demonstrate a significant dose-dependent increase in apoptotic cells in individual treatment with either agent for 48 hours. Enrichment factor was measured using subtraction of background signal. $^*P < 0.05$ relative to control. Apoptotic morphological changes such as abnormal nuclear morphology, reduction in cell number with apoptotic body formation, and cell shrinkage were observed in a dose responsive manner on 48 hour treatment with (c) curcumin or (d) garcinol. BxPC-3 and Panc-1 cells were fixed with DAPI stain and visualized using fluorescence microscopy with an excitation maximum at 358 nm and emission maximum at 461 nm.

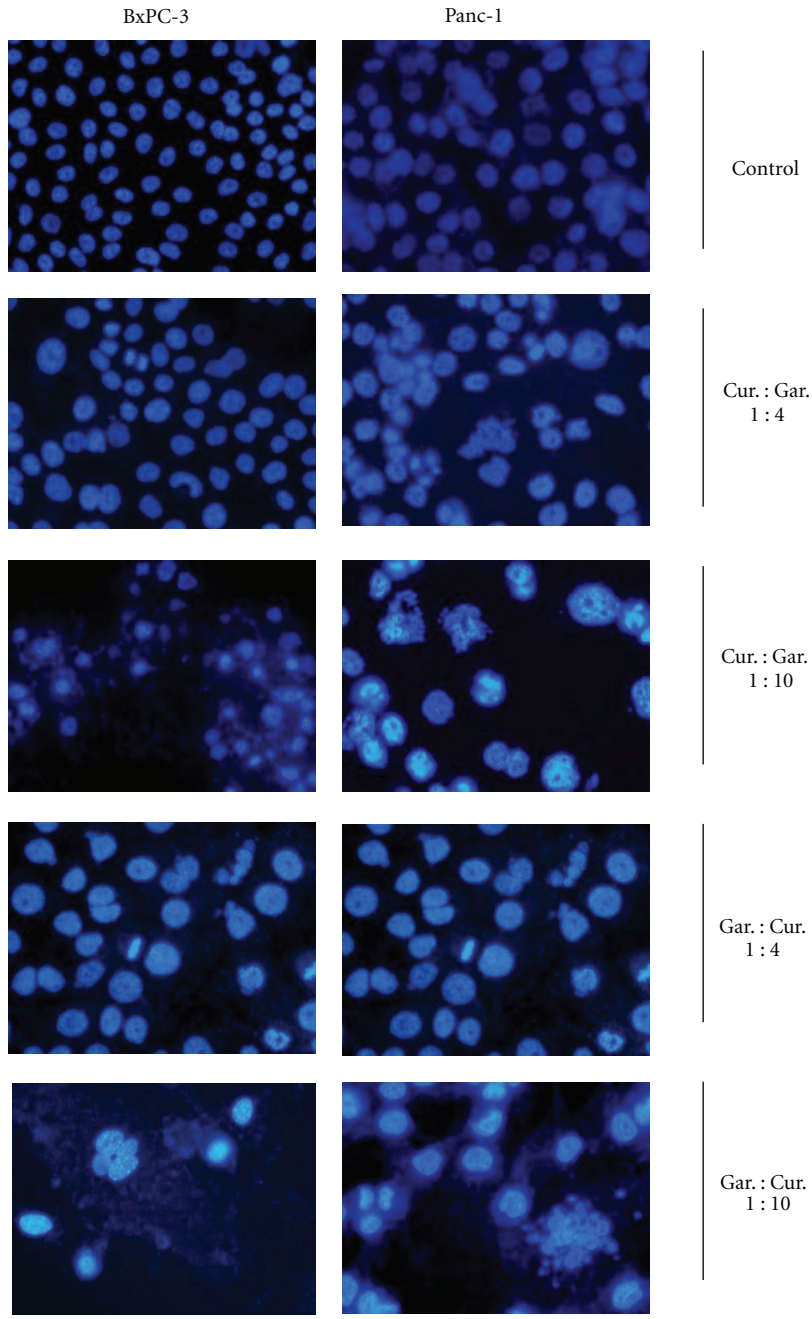

Figure 3: Apoptotic morphological changes such as abnormal nuclear morphology, reduction in cell number with apoptotic body formation, and cell shrinkage induced by combination treatment with curcumin and garcinol in different ratios for 48 hours were observed using DAPI stain in both PaCa cell lines: BxPC-3 (left panel) and Panc-1 (right panel). (1 : 4 ratio is 2.5 μM : 10 μM concentration and 1 : 10 ratio is 2 μM : 20 μM respective concentrations).

Both BxPC-3 (Figure 3 left panel) and Panc-1 (Figure 3 right panel) were subjected to different concentrations of curcumin and garcinol individually, and reduction in cell number along with formation of apoptotic bodies was observed as a dose-dependent effect. Figure 3 depicts the structural changes when curcumin and garcinol were given in combination in different ratios (1 : 4 ratio is 2.5 μM : 10 μM concentration and 1 : 10 ratio is 2 μM : 20 μM respective concentrations). Combination treatment had a more pronounced effect on induction of apoptosis than their respective individual treatments.

3.3. Caspase Activity. Caspases are a family of cysteine proteases that play a very important role in apoptosis. Caspase-9 is an initiator caspase that causes the cleavage of procaspase to active form, and Caspase-3 is an executioner caspase that cleaves other protein substrates triggering the process of apoptosis. We measured caspase-3 and 9 activities using

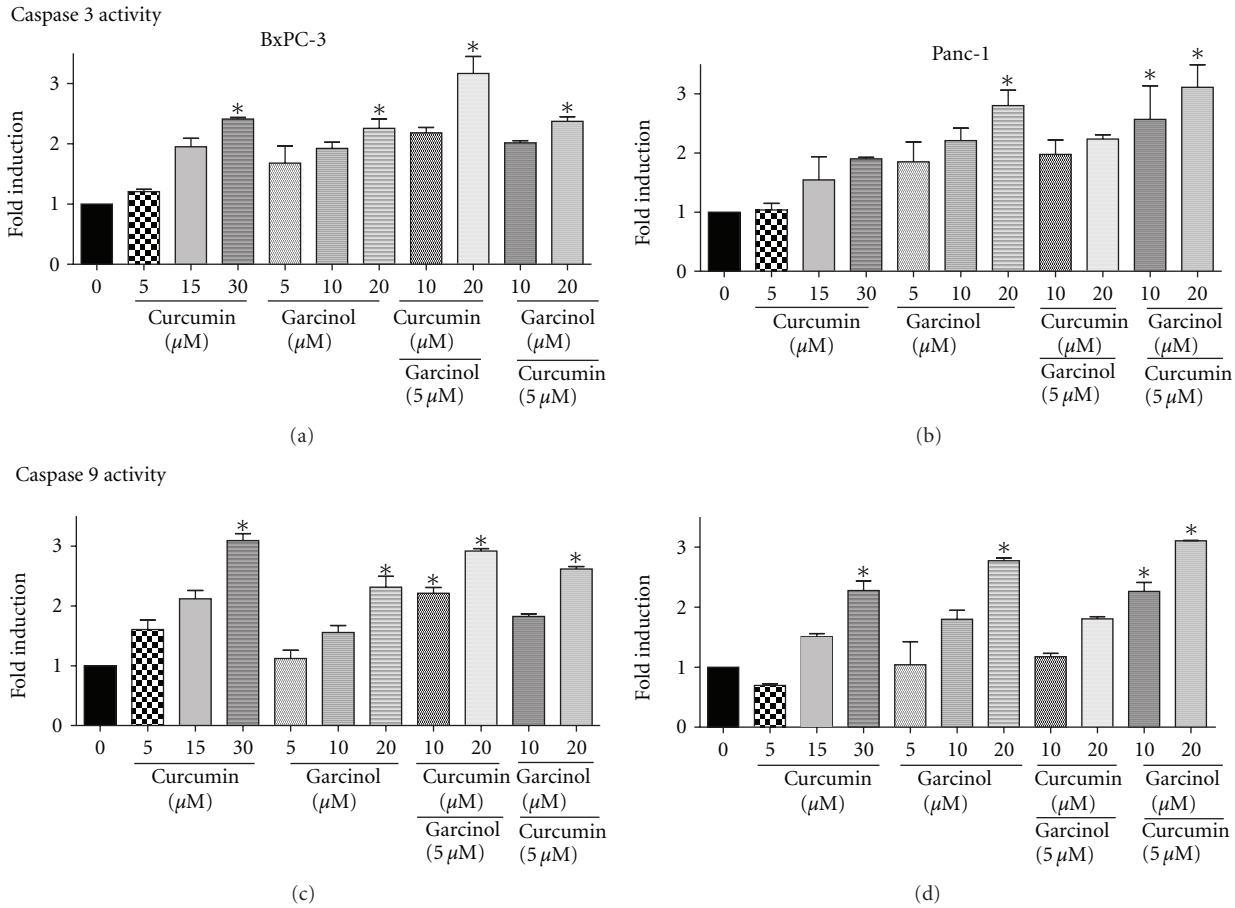

Figure 4: Garcinol and curcumin significantly increased Caspase-3 (a and b) and Caspase-9 (c and d) activity by ~2 to 3 folds in both PaCa cell lines: BxPC-3 (left panel) and Panc-1 (right panel) relative to untreated control after 48 hour treatment. Caspase activity was measured in garcinol- and/or curcumin-treated whole cell extracts using colorimetric assay. $^*P < 0.05$ relative to control.

a colorimetric assay and observed a significant 2 to 3 fold induction of active caspase-3 ($P < 0.05$) (Figures 4(a) and 4(b)) and caspase-9 ($P < 0.05$) (Figures 4(c) and 4(d)) in both Panc-1 and BxPC-3 cells upon treatment with garcinol and curcumin. Similar or higher induction was observed in combination treatment with lower doses in different ratios. Caspases are usually associated with the activity of tumor suppressor genes such as p53, p73α, or Rb. Since both the cell lines exhibit p53 mutation, activation of caspases-3 and -9 might be due to the involvement of another tumor suppressor gene, which needs to be confirmed by further analysis.

3.4. Cytotoxic Synergy. In order to determine the extent of synergism between these two agents, we tested the combination effect of garcinol and curcumin in different ratios. Cell viability reduced significantly ($P < 0.05$) upon treatment in Panc-1 cells, and the effects were more pronounced in the combinatorial approach as compared to individual doses (Figure 5(a)). Using the Isobologram analysis method, we determined the combination index values (CI) for different ratios of treatment in Panc-1 cells.

Figure 5(b) shows the different CI values obtained upon treatment. CI = 1 indicates additive effect, <1 indicates synergistic effect, and >1 suggests antagonistic effect. The CI values for ED50 (effective dose for 50% inhibition) when curcumin and garcinol were administered in the ratios of 1 : 10, 1 : 5, and 1 : 2.5 were 0.201, 0.422, and 0.659, respectively. Three combinations were tested for each ratio. *Dm* can be defined as the median-effect dose or concentration signifying the potency of the treatment. It is usually depicted in correlation with ED50 values. We observed that when Panc-1 cells were treated with only curcumin and garcinol, the *Dm* values were 26 μM and 17.65 μM, respectively. However, when curcumin and garcinol were administered in the ratios of 1 : 10, 1 : 5 and 1 : 2.5, the *Dm* values were 4.57, 8.57, and 10.79 μM, respectively. Based on the CI ED50 values and the *Dm* values, it can be concluded that there was 2 to 5 fold lower concentration requirement in combination treatment than individual treatments in order to show similar effect.

Similarly when the ratio of garcinol and curcumin was reversed, and the cells were treated with 1 : 10, 1 : 5, and 1 : 2.5 of garcinol : curcumin, the CI values at ED50 were 0.756, 0.747, and 0.921, respectively. The *Dm* values recorded for

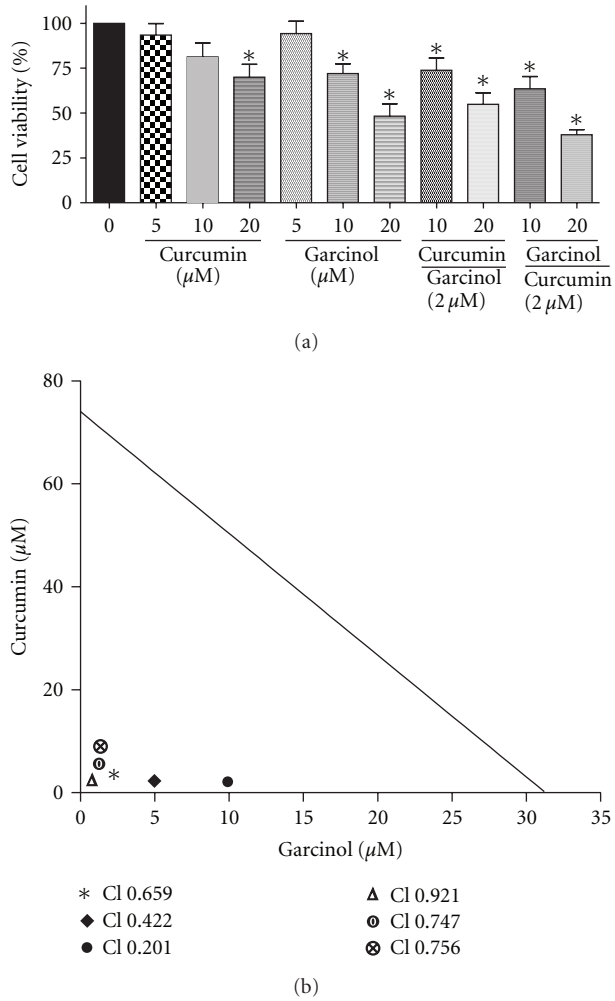

Figure 5: (a) combination effect of curcumin and garcinol on Panc-1 cell viability was determined using MTS assay. Combinatorial treatment significantly reduced cell viability more effectively than monotherapy on 48 hr treatment. $^*P < 0.05$ relative to control. (b) isobologram analysis to evaluate the extent of synergism on combining curcumin and garcinol for therapeutic effect. Combination Index (CI) values at ED50 (effective dose at which 50% cells are nonviable) depicting the synergistic efficacy of garcinol and curcumin on PaCa cell line.

the same ratios were 1.25, 2.32, and 5.11 μM, respectively. *Dm* is an important parameter which helps in establishing dose reduction leading to lowered toxicity in the host, thus retaining overall therapeutic efficacy.

Another parameter describing the sigmoidicity of the dose effect curve is the *m* value. An $m = 1$, >1, and <1 correlates with a hyperbolic, sigmoidal, and negative sigmoidal shape, respectively. We observed $m > 1$ value in all our samples except when curcumin : garcinol were in the ratio of 1 : 10 suggesting sigmoidicity of the curve. Also, *r* value is the linear correlation coefficient of the median effect plot. An *r* value equal or close to 1 indicates perfect conformity of the data. We report an *r* value close to 1 (0.97–1.00) in all our samples.

Collectively, the above results suggest that dietary interactions can be a beneficial option for the control of PaCa. This disease is a major health problem because of its increasing incidence worldwide. Given the limited therapeutic options and current unmatched clinical needs for the treatment of the patient, there is an urgent need for the development of novel agents that can influence the survival rates and quality of life for the patients. Relatively few studies have reported that the additive and synergistic effects of phytochemicals in fruits and vegetables are responsible for their potent anticancer activities. One school of thought is that this synergistic or additive effect of various bioactive compounds could be due to targeting of multiple signaling pathways. Our data clearly exhibited that garcinol in combination with curcumin had potent synergistic effect on cell viability and apoptosis.

Dietary modification is a practical approach where we can combine nontoxic phytochemicals from fruits and vegetables, and this approach may also enhance the chemotherapeutic efficacy of malignant cells with minimal toxicity to normal cells. This study demonstrates a synergistic effect between curcumin and garcinol in pancreatic cancer cells. Our findings have important implications for combination of different dietary agents for cancer therapy. However, further studies are needed to elucidate the underlying mechanisms of combinatorial approach in pancreatic cancer for enhancing efficacy and simultaneously lowering cytotoxicity to normal cells.

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Review Article

The Role of Nutraceuticals in Chemoprevention and Chemotherapy and Their Clinical Outcomes

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The genesis of cancer is often a slow process and the risk of developing cancer increases with age. Altering a diet that includes consumption of beneficial phytochemicals can influence the balance and availability of dietary chemopreventive agents. In chemopreventive approaches, foods containing chemicals that have anticancer properties can be supplemented in diets to prevent precancerous lesions from occurring. This necessitates further understanding of how phytochemicals can potently maintain healthy cells. Fortunately there is a plethora of plant-based phytochemicals although few of them are well studied in terms of their application as cancer chemopreventive and therapeutic agents. In this analysis we will examine phytochemicals that have strong chemopreventive and therapeutic properties *in vitro* as well as the design and modification of these bioactive compounds for preclinical and clinical applications. The increasing potential of combinational approaches using more than one bioactive dietary compound in chemoprevention or cancer therapy will also be evaluated. Many novel approaches to cancer prevention are on the horizon, several of which are showing great promise in saving lives in a cost-effective manner.

1. Introduction

The transformation of a normal cell into a cancerous phenotype requires stages of initiation, progression, and promotion by altering specific genes [1–3]. Although predisposition to cancer cannot be signaled out by a single factor, a group of factors place some individuals at a higher risk of acquiring the disease. Most of the high-risk cases may have a genetic background, but in some instances dietary choices can dictate the outcome of health. As determined by population and epidemiological studies, the predominant forms of cancer and cancer-related deaths are those of the lung and bronchus, breast, colorectal, and prostate [4, 5]. These cancers are also more prevalent in the western parts of the world and are much lower in Asian countries. A well-balanced diet that includes more of vegetables and fruits with less fat/meat intake is in most cases a staple of many Asian countries [4, 5]. Many hypotheses have supported that diet and environment greatly influence cellular function and health [6].

Phytochemicals are plant-based chemicals that mediate their positive health benefits directly, by affecting specific molecular targets such as genes, or indirectly as stabilized conjugates affecting metabolic pathways [7]. Many genes play

significant roles in the cell cycle pathway, and some of these are altered in cancer cells [1, 2]. The aim of most studies is to understand and formulate mechanistic pathways by which these naturally derived chemicals can alter the fate of a cell. For a cancerous cell to survive, it should be able to proliferate, obtain energy, and establish angiogenic pathways, in a tumor mass. Altering genes that affect these pathways can serve as suitable tools to decrease tumor mass and also allow for tumor regression. In this paper, the key focus will be on mechanistic pathways that are regulated by nutraceuticals to bring about changes in the tumor environment and serve as alternative approaches for cancer prevention and therapy (Figure 1).

The study of phytochemicals and the classification of these compounds have been previously reviewed [8]. However, in this paper only some of the most potent and promising chemopreventive and therapeutic molecules will be analyzed, with emphasis on combination therapy of these with other nutramolecules. Most phytochemicals derived from dietary sources are classified under an umbrella of specific chemical compounds as detailed in Table 1. These molecules may not have a nutrient value but are germane to the function of a cell. Various studies have shown that these molecules can induce apoptosis, inhibit cellular proliferation, affect angiogenesis, and affect cancer metabolism in various cancers, all of which are hindrances to tumor growth (Figure 1) [7].

Several of the phytochemicals listed in Table 1 have been investigated in terms of their curative properties. However, one must carefully interpret the observed results *in vitro* and *in vivo* before testing the same in a clinical setting. The reasons for this are manyfold. Tests in culture are pure, in that there is only one cell type in the culture plate and all conditions are controlled, including the bioactive compound. *In vivo,* however, the scenario changes as there are a host of other factors that need to be taken into account, including age, weight, diet, and metabolism of the compound. A bioactive molecule in culture may be subjected to less metabolic changes and may be presented to the cell in its native form. However, *in vivo* the same compound may be presented differently, perhaps as a conjugate, and its mode of action may change amongst the multitude of other molecules in the host's microenvironment. Many *in vivo* experiments also control for the type of diet being administered to the organism, where the concentrations or plasma availability can be adjusted. Therefore, what may work well *in vitro*, may have no agonistic effects or even antagonistic effects *in vivo,* and such discrepancies are often seen when comparing population and epidemiological studies in terms of chemical efficacy.

An effective nutraceutical is one that will have a low nontoxic dose while creating a magnitude of change in tumor dynamics. This means that at a low dose the compound should act fast on the tumor load. However, if the time taken to be effective is slow, the problems faced would be maintaining a tolerable dose and increasing bioavailability and stability. A solution to such a problem would be to use a combinatorial approach to therapy, a bioactive molecule with an effective synthetic drug or double-nutratherapy (e.g., curcumin and resveratrol). Once tumor regression sets in, dietary composition of the molecule can be adjusted.

2. Nutraceuticals and Their Preventive and Therapeutic Roles

2.1. Genistein: A Potent Isoflavone. Many phytochemicals are currently being investigated for their promising anticarcinogenic properties. *In vitro* investigations have shown that some compounds exert their antitumor functions at much higher concentrations and that dietary consumption is insufficient to achieve such effective concentrations at the tumor site. Therefore, the mode of delivery is a very important factor that needs to be considered at clinical trials and during *in vivo* studies. The nontoxic properties of natural compounds are essential to the design of a formulated therapy. However, evidence along several lines of treatment has shown that some compounds are preferentially more potent in activity when administered early in life [9, 10]. For instance, soy-based prevention of breast cancer is thought to be more successful when soy products and their derivatives are consumed in early development [9].

Isoflavones are a group of phytochemicals that are predominant constituents of a soy-based diet [9, 10]. Among isoflavones, the three major constituents that have been shown to have remarkable influences in cancer prevention and therapy are genistein, diadzein, and glycitin [11]. They are collectively grouped as phytoestrogens for their weak estrogen-like activity and bind preferentially to ER-β receptors [12–15]. Evidence of antiproliferative activity of genistein *in vitro* stems from its ability to inhibit the tyrosine kinase enzyme that is most often upregulated in cancer cells [16, 17]. As a chemopreventive agent, genistein is thought to influence the differentiation process of mammary tissue. It is believed that early differentiation of mammary tissue into terminal buds, as seen in rats, serves as a chemopreventive strategy as it reduces the susceptibility of the epithelial cells in the ducts to carcinogens or estrogen and the ontogeny process [9]. Many aggressive cancers have altered epidermal growth factor (EGF) receptors on their cell surface allowing for a continuous downstream signaling pathway for cell division [18, 19]. This is interesting, as genistein can serve as a two-fold approach molecule for prevention and treatment. When EGF binds to its receptors, tyrosine kinase activation results in the phoshorylation of tyrosine residues of proteins involved in downstream cell signaling pathways that trigger cell division. Though studies have shown that genistein increases the EGF transcript early in development of mammary tissue, this perhaps is essential for differentiation and faster development of the breast tissue. In the long run this is a positive preventive strategy of breast lesion formation in ducts [9]. However, as seen in older rats [9], EGF mRNA decreases. Therefore, a decrease in EGF mRNA coupled with inhibition of tyrosine kinase by genistein would profoundly decrease tumor growth as cell signaling pathways are crucial to tumor maintenance.

Numerous studies have highlighted the antiproliferative role of genistein in various cancers; however, there are some

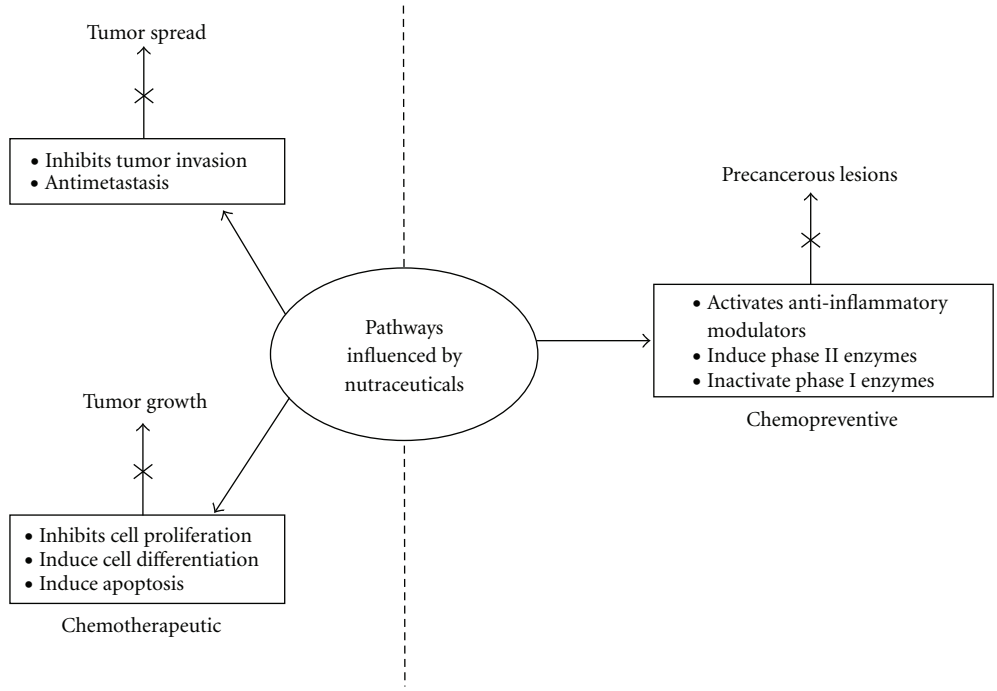

Figure 1: Cellular pathways affected by the activities of bioactive components in dietary sources. Of the natural compounds present in dietary sources, some are more involved in regulating chemopreventive pathways and some are more effective in influencing chemotherapeutic pathways. However, a few of the bioactive molecules found to date can impart both chemopreventive and therapeutic effects, such as EGCG and genistein. Compound combinations as discussed in the paper that can affect different pathways are shown and can have profound effects on tumor growth and inhibition.

Table 1: Classification of nutrients as phytochemicals and their major food source availability.

Phytochemical class	Bioactive compound	Source	*Molecular formula	Reference
Alkaloid	Caffeine	Cacao, tea, coffee	$C_8H_{10}N_4O_2$	[20]
	Theophylline	Cacao, tea, coffee	$C_7H_8N_4O_2$	
Monoterpenes	Limonene	Citrus oils from orange, lemon, mandarin, lime, and grapefruit	$C_{10}H_{16}$	[21]
Organosulfides	Allicin	Garlic	$C_6H_{10}OS_2$	[22–25]
	Indole-3-carbinol	Cabbage	$C_9H_{11}NO_2$	[26]
	Isothiocyanates	Broccoli	CNS	[27]
	Sulforaphane	Broccoli	$C_6H_{11}NOS_2$	[28]
Carotenoids	Beta-Carotene, lycopene	Tomatoes	$C_{40}H_{56}$	[29]
Flavonoids	Epigallocatechin-3-gallate	Green tea	$C_{29}H_{22}O_{15}$	[30]
	Quercetin	Black tea	$C_{15}H_{10}O_7$	[31]
	Curcumin	Turmeric	$C_{21}H_{20}O_6$	[32]
Phenolic Acids	Capsaicin	Chilli peppers	$C_{18}H_{27}NO_3$	[33]
	Ellagic acid	Black berries, raspberry	$C_{14}H_6O_8$	[34, 35]
	Gallic acid	Pomegranate, nuts	$C_7H_6O_5$	[36, 37]
Stilbenes	Pterostilbene	Blueberries and grapes	$C_{16}H_{16}O_3$	[38]
	Resveratrol	Almonds, blueberries, grapes	$C_{14}H_{12}O_3$	[39]
Isoflavones	Daidzein	Soy	$C_{15}H_{10}O_4$	[9, 40]
	Genistein	Soy	$C_{15}H_{10}O_5$	

* Molecular formulas obtained through the PUBCHEM COMPOUND Database.

studies indicating that genistein may increase cell proliferation [19, 47]. A key point to note is that nutraceuticals can be effective based on the form of genistein or its dose given at the time of the study (Tables 2 and 3), especially with respect to *in vitro* and *in vivo* models. Importantly, the downstream targets of bioactive molecules under investigation need to be ascertained for each specific tissue, if overall health applications are an issue. The nutraceutical may not affect a specific common pathway for tumors of different origins. For example, in breast tissue, EGF may be highly expressed, but, in colon cells or pancreatic cells, genes that regulate cell division other than EGF may be affected [48]. Cell culture experiments using plant-based nutrients depend on the sensitivity of the cells that are being investigated. When cell lines are established, they are derived from cancerous tissues of specific organs and are, therefore, cell-type specific. This is drastically different in a clinical setting where the molecule has to mediate its activity amongst a host of various molecules and cell types. Therefore, the concentration of the phytonutrient in the supplemented diet will be crucial to its efficacy in the tumor environment. This can help explain the discrepancies seen in clinical trials of genistein for different tissues [47, 49, 50]. Outcomes of some *in vitro* studies suggest that, like other bioactive compounds, genistein appears to have a specific cut-off concentration at which this isoflavone can exhibit anticarcinogenic activity (10 μM or even higher) [48, 51], and it is, therefore, imperative to achieve such concentrations *in vivo*.

Isoflavones, in particular, genistein, have been extensively studied as prospective antitumor molecules in the treatment of prostate cancer [19, 52, 53]. There has been a well established line of evidence that genistein works against prostate cancer, but a majority of studies are *in vitro* in cultured cells [19, 52–56]. Limited clinical trials have tested the therapeutic efficacy of genistein in prostate cancer and those that have revealed inconsistencies in cell proliferation and tumor growth [57–60]. Given the inconsistencies in some of the outcomes, emphasis should be on the dose of the supplement and the form of the nutrient in the supplement at the time of administration to the patient in clinical trials. The highest achievable plasma concentration of isoflavones is 1 μM through orally administered food sources. From previous studies, this concentration is not sufficiently significant to bring about anticarcinogenic effects on the tissue. However, there is ample evidence that genistein and other isoflavones do exhibit anticancer properties and inhibit cell proliferation and tumor growth. A clinical study by Gardner et al. [61] showed that treatment of patients with dietary supplements (82 mg/day aglycone equivalents) of isoflavone yielded a higher concentration of total isoflavones in the prostatic tissues than in serum. Therefore, there is a possibility of increasing the concentration of isoflavones to anticarcinogenic levels in tissue.

An orally administered dose of isoflavones must withstand the rigors of the alimentary canal and become metabolized before they can be made available to tissues. Most isoflavones exist as conjugates rather than in their free state. This conjugation is perhaps the best way to present the molecule to the cell in tissues, and the hydrolysis of the conjugates in the tissue allows available free genistein delivery to the cells, as presented or tested *in vitro*. For pharmaceutical companies, it is required to formulate supplements with precise ratios of individual constituents of the compound. Unless a very pure form, a capsule or supplement may contain a mixture of genistein, diadzein, and glycetin (Tables 1, 2, 3, and 4). The percentage of each nutrient in the mixture will have a profound effect on the bioavailability of the compound after metabolism (Tables 2 and 3). To design such a product is certainly not easy and is dependent on many factors, but the two essential factors are the grade/stage of the tumor and the site or origin of the tissue. Of the two isoflavones, diadzein has been shown to have a lesser apoptotic effect on prostate cancer cells but can inhibit neoplastic transformation [61]. Therefore, it would be advantageous to use supplements containing the two bioactive nutrients as chemopreventive agents.

Of the predominant high-risk cancers, genistein appears to have a greater affect on prostate cancers [52–54]. Genistein mediates the apoptosis of cancer cells by activating and/or inhibiting genes and/or enzymes germane to tumor maintenance (Figure 1, Table 4). Some of these important mechanisms are the inhibition of the activity of tyrosine kinase, nuclear factor kappa B (NF-κB), and vitamin D 24-hydroxylase [86], activation of tumor suppressor genes, and modulation of androgen-responsive gene expression, prostate-specific antigen (PSA), and the androgen receptor (Table 4). Of the prominent isoflavonones in soy, diadzein is less effective in its action on prostate cancer, but, unlike genistein, it is metabolized to equol, an isoflavandiol which has a longer half-life than genistein [87]. The longer half-life of equol creates the possibility of using this chemical in combination with other available nutraceuticals, where the net effect may be synergistic. However, prior preclinical tests are required to investigate this possibility.

Other dietary compounds are also of great interest in this regard. *In vitro*, vitamin D (Vit D) has potent tumor prevention ability and can induce differentiation and apoptosis in some of the most predominant cancers [48]. The use of nutrients as a possible treatment approach is based on the fact that chemicals occurring naturally will minimize side effects when applied to a biosystem. However, the *in vitro* dose at which Vit D induces its antitumor properties causes hypercalcemic conditions that can preclude treatment in patients [49]. In prostate cancer, a leading cause of cancer deaths in the western parts of the world, androgen ablation therapy is the choice of treatment. However, as the cancer becomes aggressive, hormone ablation therapy fails, and progression ensues via androgen-independent pathways. Therefore, alternate therapies are very much in demand. Vitamin D is an alternate form of treatment in prostate cancer (PCA) and is shown to induce apoptosis in PCA cells *in vitro*. However, all PCA cell lines *in vitro* are not equally receptive to the vitamin D treatment or genistein [88]. Cell lines such as DU145 prostate cancer cells are especially more resistant as they express high levels of CYP24, an enzyme that catabolizes Vit D3 into less active metabolites [88]. To circumvent this problem, a recent study showed that a dual combination therapy, of DU145 to genistein and Vit D3,

Table 2: Pharmacokinetic studies evaluating the bioavailability of phytochemicals at given doses.

Phyto chemical	Form	Dose	Model subject	Experimental setup	Maximum plasma concentration	Half-life (h)	Reference
Diadzein	Soy beverage	15 g Diadzein : genistein (9.27 : 10.51 mg)	Human postmenapausal women	Clinical	96.31 ng/mL	7.68	[40]
	Two soy capsules	Diadzein : genistein (7.79 : 22.57 mg)			96.02 ng/mL	6.67	
Genistein	Soy beverage	15 g Diadzein : genistein (9.27 : 10.51 mg)	Human postmenapausal women	Clinical	116.37 ng/mL	7.61	[40]
	Two soy capsules	Diadzein : genistein (7.79 : 22.57 mg)			216.84 ng/mL	7.96	
Curcumin glucoronide	Curcumoid powder form curcumin (75%), demethoxycurcumin (23%), and bisdemethoxy curcumin	10 g ($n = 6$)	Healthy human subjects (5 men and 7 women)	Clinical	2.04 ± 0.31	6.77 ± 0.83 for total curcumin conjugates	[41]
		12 g ($n = 6$)			1.40 ± 0.74		
Curcumin sulfate	Curcumoid powder form curcumin (75%), demethoxycurcumin (23%), and bisdemethoxy curcumin	10 g ($n = 6$)	Healthy human subjects (5 men and 7 women)	Clinical	1.06 ± 0.40	6.77 ± 0.83 for total curcumin conjugates	[41]
		12 g ($n = 6$)			0.87 ± 0.44		
Quercetin aglycone	Quercetin 500 plus capsule	500 mg of quercetin	Healthy human subjects (6 males and 4 female)	Clinical	15.4 ng/mL	3.47	[42]
Quercetin conjugates	Quercetin 500 plus capsule	500 mg of quercetin			336 ng/mL	Not given for plasma level, but renal clearance is 0.835	
Resveratol	Uncoated immediate-release caplets	500 mg resveratrol/caplet	Healthy human subjects	Phase I clinical test			[43, 44]
		0.5 g			72.6 (48.9)* ng/mL	2.85*	
		1.0 g			117.0 (73.1) ng/mL	8.87 (91.1)	
		2.5 g			268.0 (55.3) ng/mL	4.22 (51.6)	
		5.0 g			538.8 (72.5) ng/mL	8.52 (95.8)	
Sulforaphane	Broccoli raw	200 g	Healthy adult male subjects	Clinical	$103 \pm 31^{@}$, nM	$3.8 \pm 0.8^{@}$	[45]
	Broccoli cooked	200 g			$31 \pm 19^{@}$ nM	$4.6 \pm 0.8^{@}$	
EGCG	Beverage 200 mL	112 mg	Healthy human subjects	Clinical	Per dose (L^{-1}) $0.51 \times 10^{-3} \pm 0.08 \times 10^{-3}$	3.2 ± 2.1	[46]
D-Limonene oxygenated metabolite perillic acid	30–40 ounces of lemonade	447–596 mg D-limonene	Healthy human subjects	Clinical	2.08–13.98 μM	12–24	[21]

Table 2: Continued.

Phyto chemical	Form	Dose	Model subject	Experimental setup	Maximum plasma concentration	Half-life (h)	Reference
Lycopene	Lycopene with up to 250 mL water	10–120 mg	Healthy adult male subjects	Clinical	Range between 4.03 and 11.27 μg/dL (0.075–0.210 μM)	Range between 28.1 61.6 h	[29]

* coefficient of variation; @SD—standard deviation.

increased the sensitivity of the cells to Vit D3 by decreasing CYP24 expression. What is interesting to note is that the combination approach not only lowered the effective dose, but was able to abrogate cell proliferation as well. This lowered concentration of genistein at 100 nM is achieveable *in vivo* through dietary sources, and clinical studies would be required to determine the localization of genistein and Vit D3 in prostatic tissues.

An *in vivo* study for colorectal cancer has demonstrated a similar effect [89], but in this case the mice were given a single gavage of 250 μg of genistein. This mode of nutrient administration is useful for a preclinical test and probably has applications as a chemopreventive supplement. However, in terms of a clinical setting, patients are often exposed to a host of other nutrients or isoflavones in their diet, and; therefore, an *in vivo* model replicating such an environment with various percentages of isoflavones will allow for a better understanding of concentration and bioavailability of genistein that can mediate an apoptotic effect and reduce CYP24 expression in colonic tissues in the presence of vitamin D.

The antimetastatic properties of genistein are mediated by altering the expression of NF-κB, and inhibiting the tyrosine kinase enzyme [17, 90]. Non-small-cell lung cancer (NSCLC) is a highly aggressive form of lung cancer with a poor prognosis. Therefore, alternate approaches that drastically reduce tumor growth are of utmost importance. Activation of epidermal growth factor receptor tyrosine kinase (EGFR-TK) enhances the cell signaling pathways allowing tumor growth. The use of drugs that inhibit EGFR-TK and affect NF-κB, a gene whose transcribed products are essential for invasion and metastasis, can induce a more aggressive approach of reducing tumor size and the spread of the disease. A clinical therapy should be aimed at reducing tumor growth and spread by inhibiting mechanisms that contribute to the activation of metastasis. In NSCLC, genistein remarkably enhances the effects of EGFR-TK inhibitors, such as erlotinib and gefitinib, when used in combination with each of them, respectively. This effect was seen to be mediated by a marked reduction in NF-κB and others, such as EGFR, pAkt, COX-2, and PGE(2), essential for regulating genes that control division, proliferation and metastasis [90]. A few studies have shown how a combined approach can lower the effective dose concentration even of chemotherapeutic drugs, minimizing potential side effects. A study conducted on breast and pancreatic cells showed that, when the cells were primed with genistein, lower concentrations of the chemotherapeutic drugs were needed to significantly bring about growth inhibition and apoptosis than with the drugs alone. In addition, NF-κB was transcriptionally inhibited in the combined treatment [90].

From a number of investigations, a common thread of evidence seems to emerge that considerable variation in the efficacy of bionutrients in cancer treatment exists and differs even among the same cell lines tested. The reasons for this are manyfold (Table 5). Cell lines derived from the same tissue hypothetically should be sensitive to the same dose or chemical class of the phytonutrients, but such is not always the case. Alternate medicinal approaches have an important task to identify crucial factors that change the sensitivity of the chemical and determine chemical modifications that would be necessary to modulate more synchronized results across several cell lines expressing similar genotypic and phenotypic signatures.

2.2. Epigallocatechin-3-gallate (EGCG): A Potent Flavanol. Of the major food-derived phytochemical constituents that are extensively studied for their chemopreventive and chemotherapeutic use, EGCG and genistein are by far the most investigated. EGCG has been shown to have numerous anticancer properties which include antiangiogenic activity by affecting the transcriptional expression of vascular endothelial growth factor (VEGF) [91], inhibiting tumor initiation and promotion by inhibiting signal transduction pathways via [phosphatidyloinositol 3-kinase-Akt kinase-NF-κB] [92–94], inhibiting EGFR [95], inhibiting Her-2 receptor phophorylation in breast carcinoma cells that constitutively expresses Her-2/neu receptor [95], inducing apoptosis in estrogen receptor-(ER-) independent breast cancer cells [96], causing antimetastatic activity [97], inhibiting proteasome formation [98], inhibiting glucose-regulated protein (GRP78) activity [99]; inhibiting insulin-like growth factor-I receptor (IGF-IR) [100], and preventing invasion of tumors by inducing HMG-box transcription factor 1 (HBP1) transcriptional repressor, an inhibitor of the Wnt signaling pathway crucial for tumor-invasive property [101].

The serum level concentrations of EGCG are important to ensure that an effective response is seen without adverse or even tumor-promoting functions. Studies have shown that high doses of catechins that include a higher concentration of two prominent compounds, epicatechin gallate (ECG) and EGCG, induce hypoxia-inducible factor 1 which is responsible for activating genes related to hypoxia conditions. This allows tumor cell proliferation through alternate survival pathway mechanisms [102]. Most breast cancers are ER dependent; however, for breast cancers and others that are ER independent, EGCG inhibits the growth of tumor cells through the process of apoptosis [96, 103].

Table 3: Single-dose clinical studies evaluating the bioavailability of phytochemicals or their conjugated or active metabolites.

Phytochemical	Route of administration	Form	Bioavailability area under the curve (AUC)	AUC values	Reference
Diadzein	Oral	Soy beverage	107 ± 49.16 ng·h/mL	Adjusted to the dose	[40]
		Soy extract capsules	142.61 ± 43.94 ng·h/mL	Adjusted to the dose	[40]
Geistein	Oral	Soy beverage	121.48 ± 70.98 ng·h/mL	Adjusted to the dose	[40]
		Soy extract capsules	131.04 ± 60.79 ng·h/mL	Adjusted to the dose	[40]
Curcumin conjugates (glucoronide + sulfate)	Oral	Curcuminoid powder extract capsule form (10 g)	35.33 ± 3.78 μg·h/mL	Relative	[41]
	Oral	Curcuminoid powder extract capsule form (12 g)	26.57 ± 2.97 μg·h/mL	Relative	[41]
Quercetin aglycone	Oral	Capsule (500 mg)	62.5 ng·h/mL	Relative	[42]
Quercetin-conjugated metabolites	Oral	Capsule (500 mg)	2000 ng·h/mL	Relative	[42]
*Rersveratrol	Oral	Caplet ranging from		Relative for all	[43]
		0.5 g	223.7$^{\delta}$ ng·h/mL		
		1.0 g	544.8 (57.2) ng·h/mL		
		2.5 g	78.6 (36.2) ng·h/mL		
		5.0 g	1319 (59.1) ng·h/mL		
°Sulforaphane	Oral	200 g broccoli		Relative	[45]
		Raw	495 ± 40 nM·h		
		Cooked	286 ± 139 nM·h		
EGCG	Oral	Average 200 mL beverage	AUC$^{\kappa}$	nd	[46]
D-Limonene (perillic acid a major active metabolite of d-Limonene)	Oral	40 oz of Lemonade	5.07 to 32.59 μM·h	Relative	[62]
Lycopene	Oral	Liquid form (tomato paste)	$(AUC)_{0-96}$	Relative	[29]
		10 mg	214 ± 124.8 μg·h/dL		
		30 mg	416.4 ± 183.9 μg·h/dL		
		60 mg	421.7 ± 59.3 μg.h/dL		
		90 mg	598.9 ± 396.8 μg·h/dL		
		120 mg	655 ± 298.6 μg·h/dL		

*AUC value measured for resveratrol was AUC infinity with the coefficient of variance denoted in the brackets against the mean value.
$^{\delta}$For the lowest dose of resveratrol AUC infinity value $n = 1$.
°AUC value measured for sulforaphane was AUC0-∞.
$^{\kappa}$Based on the reference paper a list of various AUC values was given for different single doses as experimentally performed by different laboratories. Since the sample numbers were different, an average AUC value has not been given for this compound.
nd—not determined.

Table 4: Assessment of the chemotherapeutic and chemopreventive effects of nutraceuticals in combination studies.

Combination of nutraceutical	Dose used	Pathways affected or mechanistic action	Organ of study	Phase of study	Model of study	Reference
Curcumin + paclitaxel	50 μM/L + 10–50 μM/L based on the gene assessed	Inactivation of NF-κB and other metastatic genes.	Breast	*In vitro*	Human breast cancer cells MDA-MB-435	[63]
Curcumin + paclitaxel	2% w/w 10 mg/kg	Inhibition of metastasis		*In vivo*	Human breast cancer xenograft model	
Curcumin + xanthorrhizol	Synergistic effect in the range from 5 to 20 μg/mL	Induction of apoptosis	Breast	*In vitro*	Human breast MDA-MB-231 cancer cells	[64]
Curcumin + docosahexenic acid	Ratio of DHA to CCM MCF-7 55 : 30 μM MCF10A 95 : 45 μM MDA-MB 35 : 35 μM SK-BR-3 60 : 40 μM MDA-MB 50 : 25 μM	Inhibition of proliferation, more synergistic in one of the 5 cell lines tested. Enhanced uptake of curcumin by the cells. Upregulated genes involved in cell cycle arrest, apoptosis, inhibition of metastasis, and cell adhesion. Downregulated genes involved in metstasis and invasion.	Breast	*In vitro*	Human breast cancer cells SK-BR-3, MDA-MB-231, MDA-MB-361, MCF-7, and MCF10AT	[65]
Curcumin + genistein	10 μM + 25 μM 10 μM +25 μM 11 μM + 25 μM	Change in cell morphology and growth inhibition	Breast	*In vitro*	T47D and MCF-7 MDA-MB-231	[66]
Curcumin + sulphinosine	15 μM + 10 μM	Alter multidrug resistance genes. Alters the cell cycle with cells inhibited primarily in the S G2/M phase of the cycle	Lung	*In vitro*	NCI-H460/R	[67]
Curcumin + celecoxib	10–15 μM/L + 5 μM/L	Inhibition of cell proliferation and induction of apoptosis. Possible inhibition of Cox-2 pathways or through non-Cox-2 pathways	Colon	*In vitro*	HT-29 IEC-18-K-ras (Cox-2, high levels) Caco-2 (COX-2, low levels), and SW-480 (no COX-2)	[68]
Coltect + 5-aminosalicylic acid (ASA)	Coltect only 20 μM 150 mg/kg + 50 mg/kg	Inhibition of tumor growth by induction of apoptosis. Inhibits abnormal crypt formation	Colon	*In vitro* *In vivo*	HT-29 cells Chemical induction of tumors by 1,2-dimethyl hydrazine (DMH) model in rats.	[69]

Table 4: Continued.

Combination of nutraceutical	Dose used	Pathways affected or mechanistic action	Organ of study	Phase of study	Model of study	Reference
Curcumin + PEITC	25 μM + 10 μM	Additive effectives in the induction of apoptosis.	Prostate	*In vitro*	PC-3 C4 cell line	[27]
	3 μM + 2.5 μM	Inhibition of tumor growth through inhibition of Akt and NF-κB pathways.		*In vivo*	NCr-immunodeficient (nu/nu) mice bearing s.c. xenografts of PC-3 human prostate cancer cells	[70]
Pure 3—curcumin + resveratrol + EGCG; Pure 4—apigenin + baicalein + genistein + quercetin; Pure 7—Pure 3 + Pure 4; Crude 7—soy + sencha leaves + turmeric + yucca roots + saw palmetto + chamomile flowers + gingko	Individual compounds, Percentage composition in the diet not defined	Inhibit growth by inhibiting hedgehog signaling pathways.	Prostate	*In vitro*	PC-3, LnCaP and mouse cell line TRAMP-C2	[71]
		Reduce or delay the onset of tumors.		*In vivo*	Transgenic adenocarcinoma of the mouse prostate (TRAMP) mice	
D-Limonene + docetaxcel	0.2 mM + 1.9 nM	Induction of apoptosis by the regulation of proteins involved in mitochondrial apoptotic pathways	Prostate	*In vitro*	Human prostate carcinoma DU-145 and normal prostate epithelial PZ-HPV-7 cells	[72]
Tomato powder + broccoli powder (10 : 10) g/100 g of diet	11 nM of lycopene per g of diet and broccoli powder, 1.6 μM of glucoraphanin, 5.9 μM of glucobrassicin, 3.9 μM of gluconasturtiin, and 2.1 μM of neoglucobrassicin.	Reduction of tumor growth mediated by reduced cell proliferation and induction of apoptosis	Prostate	*In vivo*	Dunning R3327-H prostate adenocarcinoma model	[73]
Lycopene + ketosamine (fructose/amino acid Fru/His)	1 μM/L + 2 mM/L	Synergistic effect in inhibiting cell proliferation mediated processes. Antioxidant activity to prevent initiation of tumors.	Prostate	*In vitro*	Mat-Lylu rat cells	[74]
	20 μM/L + 5.6 mM/L	Reduce tumor growth and volume.		*In vivo*	Subcutaneous injections of Mat-Lylu cells in male Copenhagen rats	

Table 4: Continued.

Combination of nutraceutical	Dose used	Pathways affected or mechanistic action	Organ of study	Phase of study	Model of study	Reference
Lycopene + docetaxel	1 μM + 1 nM	Synergistically enhances the antiproliferative effects of docetaxel.	Prostate	*In vitro*	Human PC-3, LnCaP, DU145 cells	[75]
	15 mg/kg lycopene + 10 mg/kg docetaxel	Reduced tumor volume and growth by affecting the levels of IG-FR receptor that is highly expressed in a majority of prostate tumors. Inhibited Akt signaling and suppressed surviving necessary for tumor growth		*In vivo*	Xenograft of DU145 cells in NCR-nu/nu (nude) mice	
Quercetin chalcone (QC) and a pH-modified citrus pectin (MCP)	1.6 mg/mL + 1.6 mg/mL	Reduction in the growth of solid primary tumors	Colon	*In vivo*	Balb/c mice	[76]
Quercetin + EGCG	20 μM + 0–60 μM	Inhibits the self renewal capacity of prostate cancer stem cells (PCSCs) by synergistically inducing apoptosis decreasing cell viability in spheroids, cell migration, invasion and colony formation	Prostate	*In vitro*	Prostate cancer stem cells (PCSCs)	[31]
Resveratrol + estrogen (E2)	10 μM + 1 nM	Antagonistic estrogenic effects in suppression of progesterone receptor	Breast	*In vitro*	Human MCF-7 cells	[39]
Resveratrol + quercetin + catechin	Either all at 0.5 μM and 5 μM, or 20 μM	Synergistically inhibited cell proliferation and induced apoptosis.	Breast	*In vitro*	Human MDA-MB-231 cells	[77]
		Inhibited cell cycle progression with predominat cell cycle arrest in the G2 phase				
	0.5, 5, and 25 mg/kg body weight in a 100-μL volume	Reduced primary tumor growth and, therefore, inhibit tumor progression		*In vivo*	Breast cancer xenografts in mouse models	
Resveratrol + cyclophosphamide	50 μM + 5 mM	Inhibit cell proliferation via capase mediated cytotoxicity. Enhanced proapoptotic genes Bax, Fas and suppressed anti apoptotic gene Bcl-2	Breast	*In vitro*	MCF-7	[78]

Table 4: Continued.

Combination of nutraceutical	Dose used	Pathways affected or mechanistic action	Organ of study	Phase of study	Model of study	Reference
Resveratrol + n-Butyrate	50 μM + 2 mM/L	Inhibited cell proliferation and induced differentiation. Attentuated p27 (Kip1) levels but enhanced p21 (Waf1/Cip1) expression.	Colon	*In vitro*	Caco-2	[79]
Resveratrol + 5-Fluorouracil	200 μM + IC_{50} 800 μM	Inhibited cell proliferation and induced apoptosis by increase in capase 6 activity	Colon	*In vitro*	HCT116 p53+/+ and p53−/−	[80]
Resveratrol + genistein	250 mg/kg each in the AIN-76 diet	Suppressed prostate cancer development and mediated apoptosis by affecting the expression of steroid-receptor coactivor-3 and insulin-like growth factor-1	Prostate	*In vivo*	Simian Virus-40 T-antigen-(SV-40 Tag-) targeted probasin promoter rat model, a transgenic model of spontaneously developing prostate cancer.	[81]
Genistein + sulforaphane	5 μM/L + 15 μM/L	Affected DNA methyltransferase activity and reversed the gene expression of promoter hypermethylated genes of retinoic acid receptor h (RARb), RARB, p16INK4a p16 and O6-methylguanine methyltransferase enhanced growth inhibitory effects	Esophagous	*In vitro*	KYSE 510 cells	[82]
Sulforaphane + benzylisothio-cyanite	10 μM + 10 μM	Changed cell morphology and inhibited cell proliferation. Reduced cell viability that correlated with reduced pSTAT3 levels and an increase in PARP Cleavage	Pancreas	*In vitro*	PANC-1 cells	[83]
Sulforaphane + apigenin	10 μM + 10 μM	Synergistically induced phase II enzyme UDP-glucoronyl transferases (UGT1A1) transcript but to a lesser effect the protein level. Mediates this action by the induction of NF-κB	Colon	*In vitro*	CaCo-2	[84]

Table 4: Continued.

Combination of nutraceutical	Dose used	Pathways affected or mechanistic action	Organ of study	Phase of study	Model of study	Reference
Sulforaphane + 3,3′-diindolylmethane (DIM)	2.5 μM + 20 μM Total concentration 40 μM	Has an antagonistic effect at low concentration on cell growth. At cytotoxic concentrations of the compounds has synergistic effects on growth inhibition	Colon	*In vitro*	Human colon cancer 40–16 cell line randomly derived from HCT116 clone	[85]
Sulforaphane + dibenzoymethane (DMB)	AIN-76A diet supplemented with 300 ppm SFN and 0.5% DMB	Blocked colon tumor development	Colon	*In vivo*	Male Apc/$^{min+}$ mice	[28]

Table 5: Factors conducive to the anticarcinogenic efficacy of nutraceuticals.

Factors	Possible effects on the bioactive components in the dietary supplement
Bioavailability	Metabolism
	Time taken to achieve maximum plasma concentration
	Maximum plasma concentration, half-life
Method of ingestion	Oral
	Intraperitoneal
	Subcutaneous
Form of ingestion	Powder/capsule
	Liquid
	Cooked (solid)
	Raw (solid)
Formulation	Ratio of pure to the compound conjugates
Stability	Preference for an acidic or basic environment (pH)
Mechanism of action	Direct via receptors on the cell surface or into the nuclear region via channels
	Indirect conjugated metabolites affecting parts of metabolic pathways

As seen in MDA-MB-468 ER-negative cells, cellular apoptosis is mediated by inducing p53 and Bax proteins that enhance apoptotic functions in cells [96]. Such observations have been corroborated by *in vivo* studies using animal models [97].

Most studies have shown that anticancer properties of EGCG are mediated at higher doses. However, such doses may be irrelevant to clinical applications as they may be physiologically unachievable through dietary consumption. Therefore, clinical trials should be aimed at achieving desired anticancer preventive or tumor functions at much lowered doses. Such outcomes are possible with a dual-drug approach. One study [95] demonstrated that combining EGCG with the drug taxol, which is commonly used to treat breast carcinomas, lowered the effective dose of EGCG, ranging from 0.1–1.0 μg/mL which is a serum obtainable level through metabolism. This same group showed that higher doses (30–40 μg) of EGCG were required to mediate a similar effect when used alone [95].

EGCG can be exploited as a chemopreventive agent if it prevents cancerous lesions from occurring at lower dose concentrations and for prolonged periods of time. Most *in vitro* studies have used relatively high doses of EGCG and such doses may prove to be more tumor promotive than preventive in longer exposure time periods. In a study designed by Pianetti et al. [92], contradictory results on the effects of EGCG on Her-2/neu overexpressed receptor in NF639 breast cancer cells was observed. At short exposure times, EGCG was very effective in reducing cell proliferation, but at prolonged exposure cells became resistant to EGCG with increased levels of NF-κB. This observed change in drug-induced resistance was related to the activation of mitogen-activated protein kinase. It appears that single doses or one specific chemical constituent is mostly insufficient to induce tumor suppression or regression. Such *in vitro* data outcomes emphasize that a dual-drug treatment approach is necessary to treat the disease. This also signals that the timing of the nutradrug that is administered is important. Perhaps EGCG should be administered early in treatment, but later other phytochemicals or drugs, in conjunction with EGCG, may need to be administered in the treatment regimen. In their dual-drug treatment of NF639 Her-2/neu breast cancer cells, Yang et al. found that treating the cells initially with EGCG lowered cell proliferation and the later introduction of the MAPK inhibitor, U0126, reduced invasive phenotype [93].

Most studies determining the anticancer drug properties of EGCG are preclinical. For better understanding of specific EGCG effects, clinical trials should be carefully designed to include parameters that influence EGCG effectiveness. EGCG has different roles in ER-dependent versus ER-independent receptors, and, therefore, the type of diet needed to emulate *in vitro* doses need to be clearly understood through clinical trials and careful pharmacokinetic

Table 6: Surface receptors expressed by breast cancer cells that alter their sensitivity to treatment.

	Receptors on the surface			Cancer type	Phenotype	Reference
	ER	PR	Her2			
Breast cancer cell line						
SK-BR-3	Negative	Negative	Positive	Adenocarcinoma	Invasive	[65]
MDA-MB-231	Negative	Negative	negative	Adenocarcinoma	Invasive	[65]
MDA-MB-361	Positive	Negative	Positive	Adenocarcinoma	Metstasis	[65]
MCF-7	Positive	Positive	Negative	Adenocarcinoma	Invasive	[65]
MCF10AT	Positive	Isoform B of PR and not A	Variable	Premalignant model for cancer development	Premalignant	[65]

studies of these doses in healthy individuals, ER-positive breast cancer patients, and ER-independent tumors.

In testing phytochemicals of the same or different class it is rather uncertain which markers are necessary to determine comparable dosage values for *in vitro* versus *in vivo* efficacies. Formulation of a diet is one of the major deciding factors in the functional efficacy of a chemical constituent. It defines the concentration of the dose that will be available *in vivo*, after metabolism, and determines the diet that needs to be given to achieve such an outcome. Even though single-dose individual or mixed phytochemical treatments are currently available to cancer patients, they are relatively new and much more research in this direction is warranted. One such therapy that is rapidly gaining importance and holds promise for future cancer treatments is combination therapies using plant-based chemical compounds known as nutraceuticals.

3. Combinatorial Therapy: A Promise of the Future (See Table 3)

In prevention or treatment, combinatorial approaches can be of the following types: a phytonutrient and an effective drug, two or more phytonutrients, a synthetic phytonutrient and an effective drug, or a synthetic phytonutrient and a natural nutrient. Studies in the last few decades have focused attention on unraveling the protective properties and mechanistic actions of many phytochemicals. Still the pharmacokinetics of quite a few of these phytochemicals are not known, and, for a few that are known, there is much variability based on mode and form of delivery, dose, and the model organism of study (Tables 2, 3, and 4). Another interesting approach to enhancing curative and preventive properties of these nutrients is combination therapies. The therapy is based on the factual information available at hand and using the potent properties of one with that of another to enhance synergistic or additive actions (Figure 1). In this paper, groups that have worked with different phytomolecules belonging to a different or the same chemical class of compounds have been analyzed for their antitumorigenic activities, and the overall results of the experiments for each group are described in Table 4.

3.1. Curcumin and Taxol (See [63]). Primary breast cancer cells are commonly treated with the drug taxol. Sustained chemotherapeutic treatment with this drug has often resulted in drug resistance and tumor progression. Many chemotherapeutic drugs induce the expression of the metastatic gene NF-κB which encourages tumor progression. Interestingly, natural-based compounds that are pharmacologically safe have been shown to inactivate NF-κB expression. Taxol is a powerful drug in the treatment of cancer therefore, in order to prevent metastasis, a combination of Taxol with curcumin has been shown to downregulate the expression of NF-κB and induce apoptosis.

3.2. Curcumin and Xanthorrhizol (See [64]). A study conducted on an invasive breast tumor cell line, MDA-MB-231, has shown how and when compounds added to the cells determine the overall efficacy of the treatment. A sequential addition of curcumin and xanthorrhizol (a rhizomal sesquiterpenoid of *Curcuma xanthorrhiza*) in culture resulted in additive and antagonistic effects depending on which compound was added first to the culture. However, simultaneous addition of the compounds resulted in synergistic effects at lower concentrations and agonistic effects at higher concentrations. Such experiments provide evidence that the efficacy of a drug is dependent on dose, time, and how it is presented to the cells. Therefore, results obtained might be contradictory if doses used are simply antagonist or additive. For a successful combination therapy or prevention, synergistic doses are more relevant to mediate downstream effects, as lower concentrations of the test biomolecules will be required.

3.3. Curcumin and Docosahexaenoic Acid (DHA) (See [65]). DHA is a dietary compound present in fish oil that has been shown to have potent chemopreventive affects against cancer. Chemotherapeutic effects of compounds are often analyzed using *in vitro* models. However, what is most often observed is that all cells from the same tissue sample do not react the same way to the test compound. It is essential to have a chemopreventive or therapeutic agent that can induce its effects on a wide range of cancerous cells arising from the same tissue. In this study, the authors analyzed five cell lines

expressing different cell surface receptors (Table 6) which make them susceptible to chemotherapeutic compounds but in different ways and to different degrees. The combinatorial synergistic doses for each cell line were different, as shown in the Table 4. In particular, one breast cancer cell line, SK-BR-3, which is ER-negative exhibited a higher uptake of curcumin in the presence of DHA. DHA does not directly contribute to cell inhibition, but the combination of this compound with curcumin greatly enhances the uptake of curcumin by the cells. This compound, DHA, can reach a plasma concentration level of 200 μM. Although the focus of this study was entirely based on the SK-BR-3 cell line, the effects of reduced synergy on other cell lines in terms of transcriptome effects need to be investigated. Mammary tumors may contain a heterogenous population of cells exhibiting different surface receptors. Using combination therapy should be aimed at reducing the populations of all these cell types within the tumor site to truly exhibit antitumor potency with minimal side effects.

3.4. Curcumin and Genistein (See [66]): A Preventive Strategy. The aim to use natural compounds in diets is to render the chemopreventive properties of the compounds to the tissues. Numerous studies have shown that single dosage of compounds used alone is effective for chemoprevention. The problem faced is the inability to achieve high serum concentrations *in vivo*. Although combination studies are just beginning to surface as more prominent approaches in clinical treatment, studies, though limited, have shown that synergistic effects of the compounds are able to be achieved at much lower doses than when compounds are used alone. Especially in cancers that are hormonally regulated, the tissues are often exposed to external or internal hormonal stimulation, like estrogen, as in the case of breast tissue. Environmental agents that mimic estrogen-like activity can often stimulate or initiate the carcinogenic process. Curcumin, a curcuminoid, and genistein, an isoflavone, are derived from two different chemical classes, yet they have been known to inhibit a variety of tumor types *in vitro* and *in vivo*. Clinical trials of these compounds individually have been tested [19, 60, 104, 105]. The mechanistic action of the individual compounds in many different cancers has been investigated as well. However, using these compounds in combination drastically affects the development of tumors by mediating changes in shape and growth inhibition. Such changes were observed both in ER-positive and ER-negative cells, indicative of the dual use of such a combination in prevention and therapy.

3.5. Curcumin and Sulfinosine (SF) (See [67]). The ineffectiveness of certain drugs in prolonged chemotherapy stems from the resistance that some cancers develop with time. This is one of the major obstacles in cancer therapy, especially in cancers that are multidrug resistant (MDR). The problem in finding a suitable cure for non-small-cell lung cancers is the MDR phenotype it exhibits. Treating MDR cells such as non-small-cell lung carcinoma NCI-H460/R cells with a commonly employed drug, SF (obtained by the amination and subsequent oxidation of 6-thioguanosine), in cell cultures has been shown to inhibit cell growth. This observed cytotoxicity is enhanced several folds when low doses of the natural compound, curcumin, are used in combination, which are otherwise ineffective unless very high concentrations are used. These compounds mediate a synergistic effect in regulating the cell cycle phases and down-regulate MDR genes, thereby, enhancing tumor regression phenotypes even in the presence of mutated p53 molecules.

3.6. Curcumin and Celecoxib (See [68]). Cyclooxygenase-2 (COX-2) expression is central to the carcinogenesis of colorectal cancers. Compounds that regulate the expression or activity of COX-2 in cells may be instrumental in mediating chemotherapeutic effects on the tissue or cells. Celecoxib is a potent inhibitor of COX-2 and is presumed to target its active site. However, prolonged exposure to celecoxib results in cardiovascular problems. It appears that monotherapy regimes are very effective in inhibiting cancer growth, proliferation, metastasis, and invasion, as seen in numerous *in vitro* and *in vivo* models. However, prolonged exposures at concentrations relatively higher than what can be achieved with combination doses may result in unwanted side effects. Testing the efficacy of celecoxib with cucumin showed that at lower doses of celecoxib it was possible to enforce synergistic inhibitory growth effects on colon cells which expressed various levels of COX-2. Like many other *in vitro* investigations, this study emphasizes the fact that combining powerful drugs with naturally available potent compounds can reduce the dose needed to mediate potent anticarcinogenic effects with minimal side effects. Clinically, such studies are relevant as the doses used or needed are within the physiologically dose range. With colon cancer having such a high incidence rate in the western populations, such therapies can be taken as advantage, and biomolecules having preventive potential against the formation of precancerous lesions need to be supplemented in diets of patients at high-risk.

3.7. Coltect and 5-Aminosalicylic Acid (5-ASA) (See [69]). Coltect is a novel chemotherapeutic dietary drug with a formulation of curcumin, a turmeric extract (95% curcuminoids) mixed with turmeric powder 1 : 1, green tea (60% polyphenols and 25% EGCG) in a 2 : 1 ratio, and 0.1 mg/mL of L-selenomethionine. 5-ASA is an anti-inflammatory drug, which has been shown to have a preventive role in polyp formation that is thought to occur via the inflammation process in conditions like inflammatory bowl disorder. Coltect has been effective against HT-29 human colon adenocarcinoma grade II cells *in vitro,* and this nutraceutical complex in combination with 5-ASA has been shown to inhibit the formation or growth of chemically induced aberrant crypt foci (ACF) in rat models. The molecular mechanism by which this inhibition is mediated is via the inhibition of COX-2 pathways in HT-29 cells, which has been supported by *in vitro* studies of other groups [106, 107]. However, growth inhibition can be affected via COX-2-independent pathways possibly through mechanisms that are regulated by the functional polyphenol complex in coltect. Such complex mixtures are of clinical significance as many different control

mechanisms can be regulated by the presence of individual constituents of the polyphenols which are a part of the formulated mixture of coltect.

3.8. Phenylethylisothiocynate (PEITC) and Curcumin (See [27]). Most prostate cancers begin as a hormone-dependent tumor, and the hormone is primarily androgen. However, the more aggressive forms of prostate cancer are androgen-independent and hormonal therapies fail to be effective. Alternate therapies are, therefore, necessary to treat such aggressive forms. Most cancerous cells express various surface receptors that propagate cellular growth. Targeting such receptors can be an effective chemotherapeutic approach. Curcumin, obtained from *Curcumin longa*, has been shown to inhibit the phosphorylation of EGFR, inhibit the Akt signaling pathway, and negatively regulate NF-κB. It is an effective molecule against prostate cancer. Phenylethylisothiocyanate, a phytochemical in cruciferous vegetables, has been shown to inhibit prostate cancer cell growth *in vitro* and this observation has been supported by epidemiological studies showing that consumption of cruciferous vegetables has an inverse effect on prostate cancer risk. When two bioactive molecules with similar effects are used in treating hormonally independent tumors in affecting receptor mediated signaling, the effects are more pronounced than when used as individual compounds. With PEITC and curcumin, the observed effect was more additive than synergistic, but cell growth inhibition was profoundly affected by the inhibition of NF-κB pathways and Akt signaling pathways. Such responses were seen at lower physiological achievable doses. These results were corroborated by *in vivo* studies in mice using human PC-3 prostate cancer cells [70]. Since EGCG has similar effects on prostate cancer cells, EGCG could also possibly serve as a substitute in place of curcumin for such a treatment strategy.

4. D-Limonene and Its Combination Therapies (See Table 3)

Although a few studies have shown that D-limonene, an abundant monoterpene in citrus oils, exhibits antimitogenic activity, its alcohol-derivated perillyl alchohol (PA) has a greater inhibitory effect on cell migration in cancerous cells [108]. A study by Reddy et al. [108] used subtoxic doses of PA to determine this effect. Further preclinical studies are necessary to determine the effective yet nontoxic serum/tissue concentration that can be achieved from a diet rich in citrus intake, in conjunction with phytonutrients of the same class or a different class. Not much is known about the percentage composition of D-limonene and its metabolized constituents that are required to achieve an effective monterpene anticarcinogenic activity. In comparison to its oxygenated derivatives, limonene has the least cytotoxic effect on both noncancerous and cancerous breast cell lines and, therefore, can be applicable in chemoprevention [109].

D-limonene appears to be more effective against chemically induced colonic crypt foci [110]. These foci are preneoplastic lesions and are biomarkers for the progression into colon cancer. In colonic crypts that are chemically induced, limonene asserts its effect by inhibiting the activity of ornithine decarboxylase, an enzyme essential for the polyamine biosynthesis pathway. This pathway regulates the cell cycle, and D-limonene-dependent inhibition of ornithine decarboxylase (ODC) encourages an antiproliferative activity. If aberrant crypt foci are the initial markers for colon carcinogenesis, and D-Limonene and its derivatives assert their roles against initiation and promotion phases of cancer, then a diet rich in citrus foods can prevent crypt formation. Therefore, D-limonene appears to have potential as a chemopreventive agent in colon carcinogenesis. However, *in vivo* studies often do not correlate with results *in vitro* for many of the reasons discussed earlier. Once the intake of a compound is deemed safe for human consumption, it is imperative to analyze and study the mechanistic and metabolic functions in human subjects to determine the efficacy of the nutrient in question. As in the case of understanding limonene protection against colonic carcinogenesis, the studies were performed on rats and for shorter exposure time to the compound or its derivatives. Therefore, further *in vivo* models are required to determine the toxicity of the treatment for longer periods of time, as D-limonene is nontoxic but its alcohol derivatives could be toxic.

4.1. D-Limonene and Docetaxcel (See [72]). Many combination studies are underway to determine an effective approach in treating advanced and aggressive prostate cancers. Docetaxel, a synthetic derivative of taxol, is primarily used to inhibit the microtubular structures in cancerous cells that support cell division. In addition to its role as a microtubule disruptive molecule, it has a host of inhibitory actions on genes which regulate cell proliferation, mitotic spindle formation, transcription factors, and oncogenesis. It also upregulates genes involved in apoptosis and cell cycle progression in prostate cancer. D-Limonene, discussed earlier has been shown to have anti-prostate carcinogenic effects at low dose concentrations. Logically; therefore, combining the two compounds may have a plethora of positive antitumor functionalities. In a study by Rabi and Bishayee [72], the combined treatment enhanced the sensitivity of DU145 prostate cancer cells that are known to be apoptotic resistant. This enhanced sensitivity was thought to be mediated by reactive oxygen species (ROS) generation and activation of caspase 3 and 9. Such a positive *in vitro* outcome warrants further investigations *in vivo*, in models that mimic the progression of the disease, before it can be used in dietary supplements for therapy.

4.2. Lycopene and Fru/His (See [74, 111–114]). Serum lycopene (a carotenoid) levels have been shown to have an inverse correlation with prostate cancer risk. A diet-based population study showed that, of all the carotenoids assessed, high serum lycopene levels showed a statistically significant lower prostate cancer risk. Further analysis of their data revealed that lower serum lycopene levels in conjunction with β-carotene supplements were effective against lowering the risk of prostate cancer, suggestive for a combinatorial therapy [111]. Certain dietary compounds

can be the source of cancer formation as seen with prostate cancer. It is believed that the nonfat portion of milk and excess calcium are some main factors in prostate cancer risk [112]. Numerous *in vitro* studies have shown that carotenoids have a greater influence in reducing tumors of the prostate origin, and lycopene and 1,2-dihydroxyvitamin D3 are at the forefront as risk reduction factors. In addition to their role as potent inhibitors of prostate cancer growth, they are biologically safe and cheaper forms of treatment. 1,2-dihydroxyvitamin D3 and lycopene have physiologically different roles, but combined they modulate pathways to synergistically inhibit proliferation and differentiation at much lower concentrations [113] and bear additive effects on cell cycle progression.

The assessments that lycopene is a safe dietary molecule with anticancer properties is supported by a number of population epidemiological and cohort-based studies [112]. However, it is important to ensure that the statistical models used are able to adjust for many parameters for a true significant outcome. Regardless of the statistical model employed in these assessment studies, lycopene has emerged as a potent risk-reducing factor of prostate cancer and has been even supported by a study that was carried out across 28 countries. Intervention combination studies have not yet been performed. However, *in vivo*-based studies in mice models have shown that lycopene administered in the form of tomato powder and broccoli powder in a 10 : 10 ratio, increases its serum concentration to about 538 nM/g with about 0.4 nM/g concentrated in the prostate tissue itself. Diet-based intervention studies are required to determine the formulated diet required to improve the availability in the serum of patients and enhance the localized concentration of the molecule in the tissue. Such a diet-based treatment may serve as a suitable chemopreventive approach against prostate cancer or with patients at high-risk of the disease. Even though bioactive molecules successfully work in administering their protective functions *in vitro*, it appears through *in vivo* studies that diet and availability crucially dictate outcomes. A critical question to be asked is what factors constitute a perfect blend of bioactive mixtures. With the current research thus far, it is hard to address what the cut-off ratios are that need to be used in a diet that contain mixtures of potent nutraceuticals to coordinate similar effects clinically. Possibly a slight change in concentration of even one of the effective biomolecules may render the mixture ineffective in its function. It is rather an important task for pharmaceutical chemists and nutritionists to determine the ratios of effective biomolecules in a mixture and determine the pharmacokinetics and dynamics of that mixture.

Fru/His, a ketosamine, is also a derived product from tomatoes obtained by the reaction of a carbohydrate with an amino acid. This particular ketosamine has been found to assert chemopreventive effects by synergistically enhancing the activity of lycopene, by neutralizing ROS species and inhibiting DNA damage. Therefore, the complex of these two molecules may have a pivotal role in prostate cancer prevention. Although a rat model was used to determine the results of the treatment and pharmacokinetics of the compound are still not known, the combination of the two seemed to preferentially localize in the prostate more than in other tissues that were tested [74].

Occasionally, a combination may fail to incite anticarcinogenic effects as was seen by Mossine et al. [114]. Their experiments were conducted on the prostate adenocarcinoma rat model that was used by other groups, and their data had contradictory results to the effective action of lycopene itself and in conjunction with other micronutrients. Their study revealed that lycopene was not able to inhibit or reduce tumor load alone or in combination and that selenium alone in the mixture was able to induce antitumorigenic effects. Such outcomes are important as they open up more questions as to why a molecule that affects a given pathway behaves differently when tested within the same experimental model. Is it always dose or concentration or does molecule preparation and delivery impart effects on the efficacy of a drug?

4.3. Lycopene and Docetaxel (See [75]). Docetaxel is a potent chemotherapeutic drug that is clinically used to treat patients with advanced metastatic prostate cancers. Although the drug extends survival, it is for a very limited time period and with a poor prognosis. Lycopene, a natural compound, has been shown to have strong cancer inhibitory properties against the prostate tissue. One study tested the possibility to use this combination of compounds to enhance survival of patients that were detected with aggressive, androgen-independent tumors. As predicted, docetaxel inhibited tumor growth in nude mice that bore tumor xenografts of human DU 145 cells. Analysis of molecular mechanisms revealed that the action of docetaxel was on regulating the insulin-like growth factor receptor (IGFR) pathway by suppressing IGF, and this effect was synergistically enhanced in the presence of lycopene. Together the molecules asserted negative downstream effects on Akt signaling pathways and suppressed survivin, products of which have been known to maintain tumor growth and enhance metastasis. Clinical trials using this combination may prove effective in treating patients that express high levels of IGFR in the prostate tumor and extend survival for a longer duration than what is possibly achieved by docetaxel alone, which is about 18–20 months.

5. EGCG and Quercetin (See [31])

EGCG exhibits strong chemopreventive and therapeutic activities as it influences many pathways as shown in Figure 1. Some of the mechanistic pathways are involved in regulating the levels of Bcl2, survivin, and XIAP and activation of caspase-3/7 to induce apoptosis. EGCG is also involved in inhibiting genes that are required for transition from epithelial to mesenchymal cells and retards migration and invasion which are primarily advantageous in terms of controlling aggressive tumors. EGCG mediates such synergistic actions in conjunction with quercetin to retard the self-renewal properties of cancer stem cells (CSCs), a population that, if inhibited, can influence tumor regression. Quercetin, a polyphenol, downregulates the expression of the

heat shock protein (Hsp90) known to influence apoptosis and growth inhibition of prostate tumors. Therefore, the combination of these molecules modulates their respective therapeutic effects to mediate synergistic growth retardation of CSCs. The study by Tang et al. [31] used relatively higher concentrations of EGCG (60 μM) in the presence of 20 μM quercetin. Probably concentrations of EGCG that can mediate similar synergistic levels, albeit at lower doses, need to be investigated, and the therapeutic potential across cancer stems cells of other origins need to be assessed if clinical applications are to be considered.

6. Resveratrol and Estrogen (See [39])

Selective estrogen receptor modulators that are used in the clinical treatment of breast cancers display dual agonist/antagonist effects in the tissues, especially in cancer initiation and progression. Drugs like tamoxifen emulate antagonistic effects on estrogen to contain the tumor. Agonistic-estrogen-like activity can in some instances enhance tumor progression which is not desired in most clinical treatments. Resveratrol, a polyphenolic compound abundant in grape skin and grape products including wine, is known to have chemopreventive properties as supported by numerous *in vitro* and *in vivo* studies. However, based on the experimental cell type, resveratrol induces either agonistic or antagonistic effects that can be weak or very pronounced. Resveratrol agonistic effects are totally reversed in the presence of estrogen, possibly mediated through estrogen receptor β. This reversal of effects is pertinent to prevention of breast cancer lesions in ducts that could become long-term neoplastic and cancerous. Of its many cancer protective functions, resveratrol in combination with glucan are potent immunomodulators by upregulating Cdc42 expression [115]. When natural compounds exhibit multi-chemopreventive properties, conjugation therapies are advantageous over monotherapies. Albeit not clinically tested, harnessing cancer preventive and immune modulating functions of nutraceuticals seems to be a plausible approach to targeting hormonally independent aggressive tumors.

6.1. Resveratrol, Quercetin and Catechin (See Table 4 and [77]). The protective functions of polyphenols are manyfold. Numerous studies have analyzed their protective and therapeutic functions *in vitro* on tumor initiation that was chemically induced or *in vivo* via cellular implanted tumor formation. Few studies have established the functions of combined polyphenols on established tumors, as the majority of investigations have focused on individual mechanistic effects of the compounds. Dietary serum concentrations are influenced by the individual percentage of biomolecules present in the diet. Therefore, individual protective assessments of a compound show higher dose requirements, whilst mixtures may require lower doses to achieve the same effects. Additive and synergistic effects of compounds occur if their individual functions are enhanced in the presence of other molecules, perhaps by reinforcing the serum stability and availability of the various compounds in the mixture. Such observations were seen in both *in vitro* and *in vivo* testing of a mixture of three polyphenols, resveratrol, quercetin, and catechin, albeit pharmacokinetics studies are warranted.

6.2. Resveratrol and Cyclophosphamide (See [78]). Cyclophosphamide, a neoplastic drug, has a broad spectrum of activity on a variety of cancers, including breast cancers. The shortfall of the drug is its myriad of toxic effects on other systems. Dose reduction of the compound would be a means of reducing its toxicity without compensating its anticarcinogenic activity. Resveratrol has been shown to successfully lower the effective dose of cyclophosphamide without altering its anticarcinogenic activity. Both of the compounds together synergistically enhance caspase-mediated cytotoxic activity, as demonstrated in MCF-7 cells, an aggressive breast cancer cell line (Table 4). The combination therapy resulted in the upregulation of p53, proapoptotic genes, Bax and Fas, and downregulation of antiapoptotic gene Bcl-2, suggestive of an apoptotic mechanism involved in cell death.

6.3. Resveratrol and n-Butyrate (See [79]). n-Butyrate is a short chain fatty acid produced by bacterial fermentation of fiber in the colon. The compound is a known differentiating agent and induces an epithelial phenotype in certain cultured cells. n-butyrate is a potent histone deacetylase (HDAC) inhibitor as well and one of its differentiation-inducing properties stems from its ability to inhibit HDACs. Resveratrol, discussed above, induces apoptosis through other mechanistic pathways. The combination of two bioactive molecules influencing apoptosis via different mechanistic pathways may associate to render an apoptotic phenotype in cancerous cells and inhibit tumor formation and progression. The 2 mM dose of n-butyrate used in the Wolter and Stein study [79] is probably much higher than what can be physiologically achieved. This dose is probably suitable for treatment of colon cancers where higher molar doses of n-butyrate are possible. However, n-butyrate is highly unstable, and its serum concentrations are lower than 2 mM. Since this molecule is a differentiating agent, its clinical use in treatment of other cancers is relevant. However, such therapies require absolute lower effective doses and can probably be achieved by combining with molecules other than resveratrol or modifying the compound to specific conjugates to reach serum concentration levels.

6.4. Resveratrol and 5-Fluorouracil (5-FU) (See [80]). 5-fluorouracil inhibits thymidylate synthase, prevents DNA proliferation, and induces DNA damage-related apoptosis in colon cancer cells. Phase I clinical trials using a combination of resveratrol and grape powder have shown that resveratrol at low doses inhibit *Wnt*, a gene that is upregulated in colon cancers. Taking advantage of therapeutic effects of nutraceuticals, combined therapy of aforementioned resveratrol with 5-FU surfaces as a principal strategy in treating colon cancers. When used in combination, the presumption is that either additive or synergistic effects of the two could mediate tumor inhibition by modulating their individual apoptotic effects. The concern in using resveratrol is that higher concentrations of the doses are required in the

treatment which clinically may not be reached through dietary consumption.

6.5. Genistein and Resveratrol (See [81]). Genistein and resveratrol as individual phytochemicals are very effective in the treatment and prevention of prostate cancer progression in rodent studies. Poorly differentiated prostate cancers often fail to respond to androgen-dependent treatments, and alternate treatments are required. Androgen receptors likewise have two functional roles, one as a tumor suppressor in normal prostate tissue and the other as an oncogene in neoplastic transformation, where it is altered either by mutations or DNA damage. Genistein and resveratrol used in an *in vivo* rat-based study, modeled to understand the mechanistic action of combined treatments in the progression of prostate cancer, showed that they had more pronounced effects, albeit not synergistic. The statistically significant additive functions of reducing cell proliferation through mechanisms that regulate the androgen receptor levels and IGF-1, a biomarker found in patient serums with progressive and aggressive prostate cancers were achieved in combined therapies over the monotherapy regimes. Interestingly, the combination of genistein and resveratrol increased serum availability of both, but higher concentrations of resveratrol were achievable as compared to the single-dose regimen. Perhaps, absorption and stability of resveratrol were profoundly affected in a combined environment, which is clinically a clear advantage. The doses used in the study are physiologically safe and achievable *in vivo* by consumption of a soy-based diet high in the percentage of genistein. However, resveratrol is found in low levels in grape-based dietary products, and, therefore; a pure supplement of the compound is necessary in case that higher doses are required.

7. Genistein and Sulforaphane (SFN) (See [82])

Previous studies have shown that EGCG, a major polyphenol in green tea, can inhibit tumor growth through mechanisms that alter DNA methylation activity, reversing the expression of silenced genes involved in tumor inhibition in cancer cells. Hypomethylation of the promoters that are CpG-rich is more likely to be transcribed, with an exception of few like *hTERT*, the regulatory gene of telomerase [82, 116–119]. Epigenetics is a mechanism that has been studied for decades, and factors that regulate epigenetics are now believed to be very important as treatment possibilities in controlling tumors. DNA methylation and histone deacetylation are well known epigenetic mechanisms that regulate many of the genes involved in cancers of various origins. Genistein combined with SFN, an histone deacetylase inhibitor (HDACI) has been successful in inducing the transcription of genes involved in regulating cell cycle by reversing the hypermethylated states of their promoters. This change was observed at low doses and was enhanced in the presence of sulforaphane more than that when genistein was used alone. However, *in vivo* studies of the same are warranted to determine epigenetic behavior of the dietary compounds before applications to human treatments are considered.

7.1. Benzylisothiocynate (BITC) and SFN (See [83]). BITC and sulforaphane are ITCs derived from cruciferous vegetables like broccoli. Individually both these molecules exert potent chemotherapeutic properties strongly supported by numerous studies. Oddly, even though both are isothiocyanates, they exert their therapeutic effects by controlling different pathways involved in tumorigenic inhibition. STAT3, a member of the STAT group of transcriptional factors, is required for early development and is dispensable in adult tissues. However, there appears to be a correlation between the constitutive expression of STAT3 and tumor development, indicative of its role as an oncogene. This gene appears to have important roles in cell proliferation, angiogenesis, and metastsis, a crucial requirement of tumor survival. Both BITC and sulforaphane have cancer inhibitory effects, affecting independent cell signaling pathways. However, the sequential combination of the two has been shown to regulate the STAT3 gene and others (Table 4), thereby, inducing apoptosis. How dietary molecules are presented to the cells *in vitro* is important to its cellular mechanistic actions. In the study by Hutzen et al. [83], sequential addition of BITC to the cells after sulforaphane treatment was performed, which enhanced the reduction of STAT3 levels; however, simultaneous additions were not performed. Simultaneous additions would be important for any combination study to determine possible synergistic, additive, or antagonistic effects between the compounds. Preclinical studies should include various combinatorial interactions of the nutraceuticals being tested to determine the best way of using combined molecules in therapy.

7.2. SFN and Apigenin (See [84]). Phase I and Phase II enzymes are extremely important to cancer prevention. Dietary foods are sometimes modified to produce carcinogens through metabolism by the action of Phase I enzymes. Subsequently, the action of Phase II enzymes rapidly metabolizes these products to more soluble forms that are eliminated as body waste. Phase II enzymes are more concentrated in the duodenum and small intestine and less available in the colon. Increasing the availability of these enzymes in the colon can get rid of harmful carcinogens reducing the incidence of colon cancers, and, therefore, dietary supplements that induce Phase II enzymes would be promising tools for colon cancer prevention. SFN, an isothiocyanate, and apigenin, a flavanol, have independent cancer preventive functions. SFN is a strong inducer of UDP-27 glucuronyltransferase (UGT1A1). UGT1A1 is a major player in the detoxification process of carcinogens formed in the body and, therefore, is a potent Phase II enzyme. Treating nondifferentiated colon cells with a combination of SFN and apigenin was found to synergistically induce the expression of UGT1A1 suggesting a possible dietary tool for colon cancer prevention. The *in vitro* dose of the individual compounds used in the study was at physiological safe levels and can be easily achieved *in vivo.*

7.3. SFN and 3,3′-Diindolylmethane (DIM) (See [85]). The importance of investigating the roles of dose combinations on chemopreventive or therapeutic functions has been well

dissected in a study by Pappa et al. [85]. Lower doses of SFN demonstrate antagonistic effects on cell proliferation and higher doses of both compounds had synergistic effects. Synergism of compounds is preferred if the outcome is tumor regression, but in clinical treatments synergistic actions should be mediated at safe lower concentrations rather than at cytotoxic levels. Presumably, the choice of compounds used, based on the genetic or cellular function required, is imperative to the success of the treatment. Possibly, SFN can synergistically inhibit the proliferation of cancer cells with compounds other than DIM at much lower doses, which has been investigated in studies using SFN with flavanols. This clearly highlights the problems of using combined therapies, especially since dosage is of critical importance for the success of clinical trials.

7.4. SFN and Dibenzoylmethane (DMB) (See [28]). When seeking for dietary molecules with potential chemoprotective and therapeutic properties, it is essential to understand how they mediate their combined action. Based on mechanistic studies, only compounds that are able to achieve synergistic or additive inhibitory or inductive actions on cellular genes, pathways, and/or phenotypes can then be chosen for treatment, even though their individual actions may be more pronounced. DMB is antimutagenic. Patients with aberrant polyp crypt (Apc) mutations are prone to spontaneously form aberrant polyps in their intestinal tissue, which later can transform to colorectal cancers. Treatment with DMB found in licorice can effectively inhibit such mutations in Apc, thereby protecting individuals from aberrant polyp formations. This molecule, therefore, has potential in terms of colon cancer prevention.

SFN has a myriad of chemopreventive functions as seen before in other studies and in various tissues. A combination of these two chemopreventive agents will have a profound impact on individuals that are at high-risk or reduce the incidence of colon cancers through dietary supplementation. The study by Shen et al. [28] showed that dietary intake of SFN and DMB negatively influenced the incidence and number of tumors formed in the Apc mice. The combined doses used were half that of the individual doses. However, the observed effects were still synergistic at these doses. Interestingly, the serum and plasma levels of SFN and DBM were lower in the combined doses than when the compounds were used individually. Regardless of the low serum availability, the combination was able to mediate synergistic tumor inhibitory effects. This has important clinical significance as it is possible to achieve greater tumor toxic effects at low plasma concentrations. Mechanisms that influence such actions at low serum availability need to be further investigated.

8. Future Directions

Chemopreventive agents are much sought after as an early interventional approach to prevent tumor development or to lower the incidence risk of cancers. Given that the current available methods of treatment are chemotherapy, radiation, and surgery, all of which can induce significant side effects, an urgent need for alternate or adjuvant therapies has arisen. Phytochemicals are relatively safe and abundantly available from dietary sources. Therefore, alternate medicine aims at harnessing the protective properties of these nonessential nutrients toward cancer prevention and treatment. A large database of studies supports the use of biomolecules in cancer treatment, albeit a majority of those are *in vitro* studies. Regardless of limited *in vivo* studies and clinical trials, phytochemicals show great promise in cancer treatment considering their safe use. Caution must be taken when addressing the efficacy of these molecules in clinical trials as many factors modulate their effects on cellular functions as detailed in Table 5. Combinatorial studies also show great promise, especially when lower nontoxic doses are required for prolonged periods to mediate potent chemotherapeutic functions with minimal side effects. Two of the major problems currently faced are dosage and delivery. To maintain a constant physiological serum dose availability, it is imperative that the agent is concentrated and stable in the tissue of concern. Combination technologies may be a solution to this problem. Nanotechnology is fast catching pace as the next level of technology in all spheres of science. Limited *in vitro* studies have shown that encapsulating dietary supplements in nanoparticles can effectively deliver the supplement and increase its stability and availability. Perhaps research needs to focus on such possibilities as avenues of using combination therapies.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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