Special Article

Chemopreventive properties of 3,3′-diindolylmethane in breast cancer: evidence from experimental and human studies

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Diet is a modifiable factor associated with the risk of several cancers, with convincing evidence showing a link between diet and breast cancer. The role of bioactive compounds of food origin, including those found in cruciferous vegetables, is an active area of research in cancer chemoprevention. This review focuses on 3,3′-diindolylmethane (DIM), the major bioactive indole in crucifers. Research of the cancer-preventive activity of DIM has yielded basic mechanistic, animal, and human trial data. Further, this body of evidence is largely supported by observational studies. Bioactive DIM has demonstrated chemopreventive activity in all stages of breast cancer carcinogenesis. This review describes current evidence related to the metabolism and mechanisms of DIM involved in the prevention of breast cancer. Importantly, this review also focuses on current evidence from human observational and intervention trials that have contributed to a greater understanding of exposure estimates that will inform recommendations for DIM intake.

INTRODUCTION

Cruciferous vegetables have been shown to be protective against breast cancer in some,[1–8] but not all,[9–11] epidemiological studies. Discrepancies in current findings are thought to be explained in part by variances in exposure to multiple bioactive constituents found in this unique classification of vegetables. In fact, the chemopreventive roles of multiple phytochemicals found in crucifers have been described previously.[12] The argument for the focus on one bioactive constituent in particular, 3,3′-diindolylmethane (DIM), as a relevant bioactive food compound in breast cancer chemoprevention is based on the extensive evaluation of DIM in relation to its chemopreventive potential, particularly for breast cancer. DIM is one of the best-characterized and most abundant bioactive compounds found in commonly consumed crucifers. The purpose of this review is to describe the metabolism of DIM, the mechanisms of action of DIM against breast cancer, and the current state of human observational and intervention trials with DIM to introduce the next steps toward advancing understanding and developing guidance on DIM intake for public health.

METABOLISM OF DIINDOLYLMETHANE FROM INDOLE-3-CARBINOL

DIM is readily metabolized in cruciferous vegetables (Figure 1). Cruciferous vegetables are within the mustard family, or the family Brassicaceae (Cruciferae). Commonly consumed cruciferous vegetables include broccoli, bok choy, cabbage, cauliflower, collards, kale, Brussels sprouts, and kohlrabi. Once consumed,

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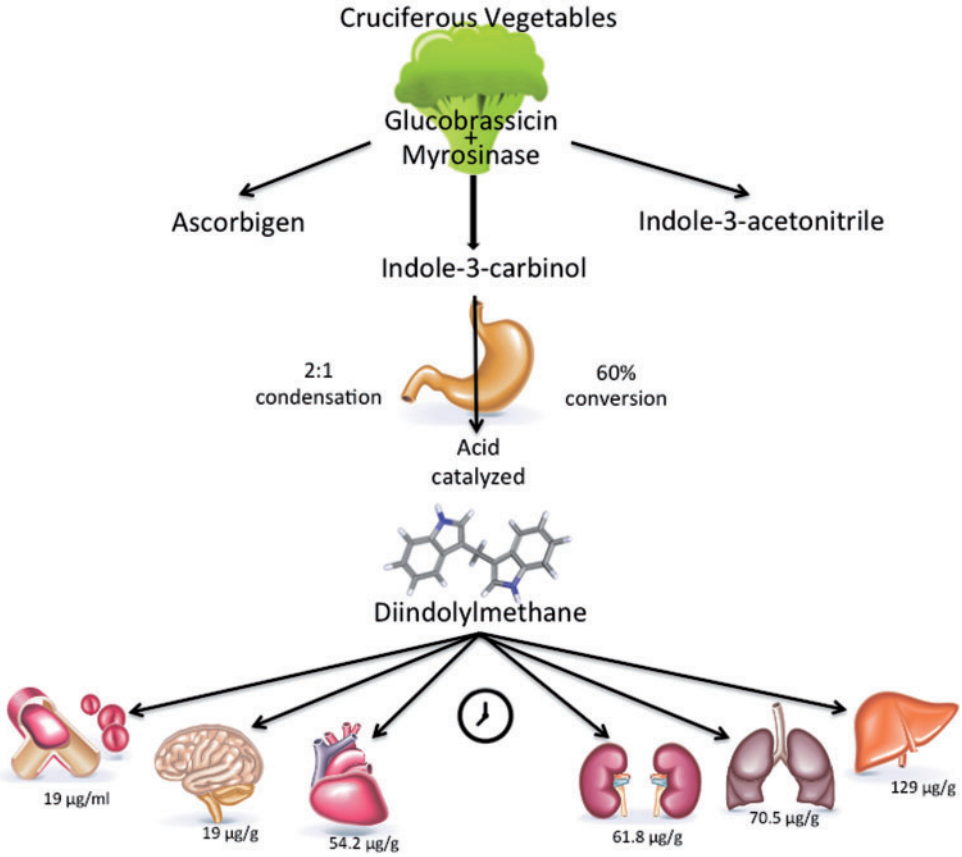

Figure 1 **Metabolism of diindolylmethane from cruciferous vegetables.** Diindolylmethane is an end product of the pH-dependent metabolism of indole-3-carbinol. The concentration of diindolylmethane is highest in liver, followed by lung, kidney, and heart and, to a lesser extent, brain and plasma. Concentrations are time dependent, as demonstrated in a mouse model after supplementation with pure crystalline DIM at a dosage of 250 mg/kg.[26]

bioactive constituents are rapidly metabolized into several intermediate and end products. The bioactive content and end products vary and are dependent on the specific vegetable consumed. Other factors that influence DIM content in cruciferous vegetables include plant age, cultivar, and vegetable storage and preparation methods.[13,14] The spectrum of effects from cooking and storage on DIM concentrations remains relatively unknown and warrants further exploration.

Cruciferous vegetables contain bioactive precursor compounds known as glucosinolates (Table 1).[15–19] Major glucosinolates are glucobrassicin and glucoraphanin, the latter of which is a derivative of isothiocyanates,[20] including sulforaphane. The average human consumption of glucosinolates from food sources is estimated at 0.5μM/kg/d.[12] US dietary intake estimates for cruciferous vegetables are low[10] and are currently not classified by specific vegetable type, limiting the availability of intake estimates of glucosinolates. The average consumption of glucosinolates from vegetable sources is nonspecific and approximated,[21] and estimates for US intakes are generally lower than those for European and Asian nations. Chemopreventive roles of phytochemicals have been described previously, but only limited data about the specific types of vegetables contributing to the overall glucosinolate intake in the United States are available.

Glucobrassicin is the most abundant glucosinolate in vegetables within the family Brassicaceae. Enzymatic breakdown of glucobrassicin by the plant-derived enzyme myrosinase during plant storage, preparation, and/or chewing[22] yields various indoles. Included in the various indoles is indole-3-carbinol (I3C), which is a relatively unstable compound. In fact, a pH-dependent, acid-catalyzed condensation rapidly converts I3C to oligomers that include DIM, which is the major indole bioactive compound, accounting for an estimated 60% of the I3C end product.[23] As the conversion is pH dependent, exposure to stomach acid is necessary for the conversion of I3C into DIM[22] and other acid condensation products. In experimental models, I3C has been shown to self-condense to produce DIM at a ratio of 2:1.[24]

DIM concentrations rise during cooking,[25] in part because of the thermal activation of myrosinase. This is evidenced by a 6-fold increase in DIM concentrations in boiled cabbage compared with uncooked cabbage.[25] After ingestion, DIM concentrations in different tissues

Table 1 **Sources and bioactive concentrations of glucosinolates**

Source	Glucosinolate content (mg/100 g, raw)[15]	Glucosinolate content (mg/100 g, cooked)[15]	Glucobrassicin content (mg/100 g)[16]	3-Indolylmethyl content (μmol/100 g, fresh)[19]	Isothiocyanate yield (μmol/100 g, fresh)[20]
Broccoli	61.7	37.2	–	71.7	6.9
Brussels sprouts	236.6	135.9	29.02	443.3	9.6
Cabbage	58.9	78.6	35.84	–	31.7
Cauliflower	43.2	42.0	18.29	75.9	1.5
Collard greens	200.67	–	–	150.4	5.8
Kale	89.4	69.10	92.13	69.5	3.7
Kohlrabi	45.9	73.4	5.38	27.7	–
Radish	92.5	–	1.31	6.0[18]	–
Turnip	93.0	–	–	62.0[17]	9.0

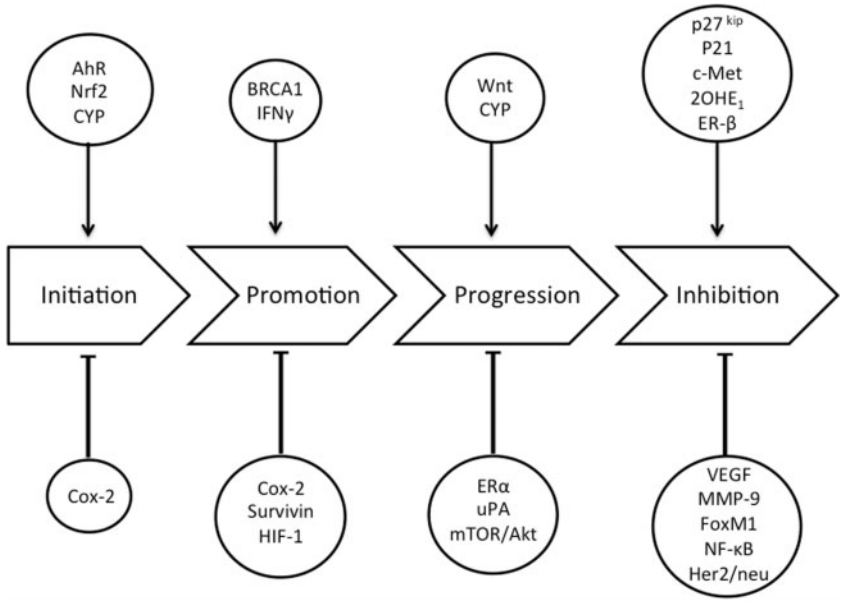

Figure 2 **Biological targets of diindolylmethane in breast carcinogenesis.** *Abbreviations*: AhR, aryl hydrocarbon receptor; Akt, protein kinase B; Cox-2, cyclooxygenase 2; CYP, cytochrome P450; ER-β, estrogen receptor β; FoxM1, Forkhead box M1; Her2/neu, human epidermal growth factor receptor 2; HIF-1, hypoxia-inducible factor 1; IFN-γ, interferon-γ; MMP-9, matrix metalloproteinase-9; mTOR, mammalian target of rapamycin; NF-κB, nuclear factor-κB; $2OHE_1$, 2-hydroxyestrone; uPA, urokinase-type plasminogen activator; VEGF, vascular epithelial growth factor.

vary. The highest concentration of DIM is found in the liver. Postprandial concentrations are also elevated in kidney, lung, and heart and, to a lesser extent, in blood plasma, and brain. However, after 24 hours, DIM is no longer measurable in brain tissue.[26] Metabolites of DIM are found in both human serum and urine but demonstrate significant clearance within 24 hours.[27,28]

MECHANISMS OF ACTION IN CELL LINES

In the 1970s, Wattenberg and Loub[29] first described the presence of DIM in crucifers, the cancer-preventive activity of freeze-dried broccoli, and the bioactivity of supplemental DIM in the prevention of carcinogen-induced breast cancer in animals. More recently, a wide array of mechanisms of cancer-related bioactivity of DIM have been described,[30] including the specific efficacy of DIM in modulating carcinogenesis at all stages of breast tumor development, including initiation, promotion, and progression (Figure 2).

Initiation of breast tumors

First, DIM can be considered an anti-initiating agent through its ability to stimulate cellular detoxification pathways. DIM is reported to modulate aryl hydrocarbon receptor (AhR), as evidenced in multiple breast cancer cell lines.[31] Aryl hydrocarbon receptor is a ubiquitous cytoplasmic receptor that, when activated and transported to the nucleus, promotes transcription of genes that stimulate the expression of detoxification enzymes, including the phase I cytochrome P450 (CYP) family. Cellular responses to AhR signaling can either promote or diminish inflammation. DIM has an

affinity to bind to the AhR.[32] Modulation of AhR by DIM treatment has also been shown to stimulate the Nrf2-mediated phase II response, which enhances excretion of genotoxins and induces a significant antioxidant response.[30,33] Through the activation of AhR and Nrf2 signaling pathways, DIM effectively increases detoxification and reduces inflammatory signaling, blocking what could otherwise be cancer-initiating events. Furthermore, modulation of AhR by DIM inhibited the growth of mammary gland cell cancer, an action suggested as a mechanism of crosstalk between estrogen receptor α and AhR.[34]

Promotion of breast tumors

The influence of DIM on the AhR results in a change in gene activity that reduces the induction and activity of the enzyme cyclooxygenase-2.[35] Evidence from mammary cell lines has demonstrated the role of DIM in reducing oxidative stress by stimulating the phosphorylation of BRCA1.[36] Further, DIM has a demonstrated role in reducing cyclooxygenase-2–induced inflammation in mammary cell lines.[35] In advanced stages of tumor development, DIM has been shown in tumor cell line models to inhibit the expression of genes involved in angiogenesis and energy metabolism, including those involved in the induction of survivin[37] and hypoxia-inducible factor-1.[38] The interferon-γ signaling pathway also is activated by DIM through the interferon-γ receptor and the interferon-γ-responsive proteins p56- and p69-oligoadenylate synthase, which inhibit growth of human breast cancer cells, resulting in inhibition of cell proliferation.[39] Additionally, DIM has been proposed to have an epigenetic effect on breast cancer. Modulation of noncoding RNA such as microRNA 21 by DIM resulted in the downregulation of CdC25A, an important protein regulating the cell cycle, and resulted in the inhibition of cell proliferation.[40]

Progression of breast tumors

The effects of DIM on transcription and proliferation are mediated by estrogen receptor α and are evident at a concentration of 1μM.[41] However, in the notable absence of estradiol, concentrations of DIM at 10μM have been shown to activate estrogen receptor α signaling pathways in human breast cancer cell lines in vitro, increasing cellular proliferation in an estradiol-independent manner, yet an opposite effect (growth arrest) can be demonstrated when higher concentrations of DIM (50μM) are provided.[42] These dose-dependent studies suggest that protective associations between DIM and breast cancer may require exposures well above what would be possible with human dietary modulation. In estrogen-dependent and estrogen-independent breast cancer cell lines (MCF-7 and MDA-MB-231, respectively), DIM has been observed to arrest proliferation, possibly through arrest of de novo cell lipogenesis or induction of Wnt signaling pathways.[43] DIM may also act as an aromatase inhibitor. It was efficient at decreasing aromatase expression in MCF-7 cells and also upregulated *CYP19* expression, which encodes aromatase and synthesizes estrogens in MDA-MB-231 cells. It has also been shown to have greater antiproliferative activity than I3C or cabbage juices in MDA-MB-231 cell lines.[44]

DIM may reduce the invasive and metastatic potential of breast tumors. In one study, MDA-MB-231 cells exposed to DIM showed a downregulation of urokinase plasminogen activator, resulting in stabilization of the membrane. The urokinase plasminogen activator–independent effects on tumor growth potential were demonstrated through possible downregulation of vascular endothelial growth factor and metalloproteinase-9, leading to inhibition of both cell growth and migration of breast cancer cells.[45] DIM has been shown to reduce the expression of both vascular endothelial growth factor and metalloproteinase-9 via downregulation of transcription factor Forkhead box M1 (FoxM1), further supporting a role for DIM in reducing breast cancer metastatic events.[46] Additional support for an antimetastatic role of DIM was demonstrated in MDA-MB-231 and MCF-7 cell lines. Administration of DIM was associated with a marked reduction in the chemokine receptor CXCR4 and its ligand, CXCL12, thus reducing signaling from breast tissue to promote metastatic growth.[47] DIM has been demonstrated to induce apoptosis in breast cancer cells MCF-7, MDA-MB-231, and MDA-MB-468 in vitro.[48]

DIM alters cancer growth through modulation of protein kinase B (Akt)-dependent bioactivity. An increase in Akt activity allows cells to evade death. In breast cancer, Akt is activated in situ,[49] and breast cancer cells rely on this pathway as a survival factor. Growth factors, including epidermal growth factor, insulin-like growth factor 1, and hepatocyte growth factor activate Akt in cells. Nicastro et al.[50] found that, after 4 hours, a concentration of 25μM DIM optimally inhibited the activation of Akt in MDA-MB-231 cells but did not inhibit the activation of Akt in nontumorigenic cells. DIM did not inhibit activation of Akt by epidermal growth factor or insulin-like growth factor 1 but did reduce activation of hepatocyte growth factor. The mechanism of this inhibition is thought to be through decreased phosphorylation and, therefore, decreased activation of c-Met, a hepatocyte growth factor receptor, at tyrosines 1234 and 1235. DIM also had inhibitory effects on a substrate of Akt, GSK-3α/β.

There are a limited number of studies describing the role of DIM in targeting mammalian target of rapamycin (mTOR), a key regulatory molecule in cell growth. Cancers with overexpression of mTOR exhibit a 3 times greater risk of recurrence.[49] One study showed DIM significantly inhibited mTOR and Akt activity in cancer cells expressing platelet-derived growth factor-D (PDGF-D).[51] This is important because inhibition of mTOR and Akt activity is correlated with decreased cell proliferation and invasion. Previous work has shown that breast cancer cell lines expressing PDGF-D, including the MDA-MB-231 and SUM-149 lines, are more invasive than those that do not express PDGF-D.[52] Inhibition of PDGF-D in these cells resulted in decreased cell proliferation and increased apoptosis. The inhibition of the mTOR pathway without activation of Akt in PDGF-D–expressing cancer cell lines further suggests the therapeutic potential of DIM. This area warrants additional research and discovery.

Inhibition of breast tumors

In combination with Taxotere, a concentration of 40μM DIM resulted in a 78% inhibition of growth and a decreased invasive capacity of the aggressive breast cancer cell line MDA-MB-231; these findings were associated with decreased activation of FoxM1. MDA-MB-231 cells express higher levels of FoxM1 than MCF-7 breast cancer cells. Cells treated with DIM showed reduced FoxM1 mRNA levels. Downregulation of FoxM1 expression induced the growth-inhibitory effect of DIM, suggesting a mechanistic role of FoxM1 and a regulatory role of DIM.[46]

DIM has been shown to induce select tumor-suppressing proteins, including p21 and $p27^{kip}$, in cell culture.[53,54] In breast cancer cell lines that overexpress both human epidermal growth factor receptor 2 (Her2) and activated Akt, DIM exposure resulted in inhibition of activated Akt expression as well as independent induction of both $p27^{kip}$ transcript expression and nuclear localization of $p27^{kip}$, ultimately resulting in apoptosis.[53] Apoptosis was also evident in Her2/neu-positive human breast cancer cells treated with a combination of DIM and paclitaxel, resulting in G_2 phase cell cycle arrest. Moreover, treatment with DIM alone decreased activation of the Her2/neu receptor, affecting cell growth and differentiation.[55]

More recently, DIM has been demonstrated to protect against ionizing radiation through activation of the protein kinase ataxia telangiectasia mutated (ATM), which regulates responses to DNA damage and oxidative stress as well as cell survival signaling through nuclear factor-κB (NF-κB).[56] Overexpression of PDGF-D is linked to increased DNA-binding activity of NF-κB in aggressive breast tumors.[52] Conversely, DIM did not protect human breast cancer xenograft tumors against radiation, suggesting its potential use to mitigate undesirable side effects of cancer treatment.[56]

Modulation of estrogen

Metabolites of sex hormones, particularly estrogens, have shown an important role in breast cancer initiation and progression. Thus, modulation of sex hormones is an active area of research. The effects of DIM on estrogen activity are thought to be primarily the result of altered CYP enzyme metabolism by DIM. Changes in steroid hormone metabolism and estrogen metabolite profiles are consequences of enhanced CYP expression.[57] In turn, changes in CYP enzyme activity and the resulting alterations in hormone metabolite concentrations modify oxidation and reduction reactions as well as the activity of estrogen, thus altering breast cancer risk. More specifically, CYP enzymes convert estrone to hydroxyestrones.[58] Of the estrogen metabolites, 2-hydroxyestrone ($2OHE_1$) has been suggested to have a protective effect against breast cancer.[59]

Expression of *CYP1* genes is low in some breast cancer cell lines. *CYP1A1* is responsible for promoting the metabolism of estrogen toward greater $2OHE_1$ production; transcripts of *CYP1A1* and *CYP1A2* are increased when exposed to DIM.[58] Importantly, DIM supplementation has been shown to enhance the 2-hydroxlyation of estrogen, resulting in selective activation of estrogen receptor β target genes, which is thought to contribute to anti-inflammatory effects in hormone-responsive cell lines.[60] The influence of DIM on AhR, as described above, results in reduced production of the carcinogenic 4-hydroxyesterone ($4OHE_1$).[61] Increased $4OHE_1$ has been associated with breast tumor formation and related to initiating mutations through formation of depurinating DNA adducts.[62] DIM also induces expression of *CYP3A4*,[63] which, similar to *CYP1A1*, has been shown to influence total production of the mitogenic metabolites $4OHE_1$ and 16α-hydroxyesterone ($16\alpha OHE_1$)[64] by 2-hydroxylation of estrogens.

The efficacy of DIM has been demonstrated in vitro in breast tumor subtypes. DIM selectively induced cell cycle arrest and apoptosis in both estrogen receptor–positive and estrogen receptor–negative breast cancer cells, without producing evidence of antiproliferative activity in normal breast epithelial cells.[53] The chemopreventive activity of DIM may have clinical applications in both hormone-dependent and hormone-independent disease. This may expand therapeutic options for triple-negative breast cancer.

MECHANISMS OF ACTION IN RODENTS

While I3C has been demonstrated to a play role in breast cancer prevention in animal models,[65] the conversion of I3C to DIM in cell culture[24] suggests that the growth-inhibitory effects of I3C on breast cancer cells are likely attributable to DIM, rather than to I3C alone. In fact, the bioavailability of supplemental DIM has been evaluated in Sprague Dawley rats. DIM was administered either at 200 mg/kg by atraumatic gavage or as a single morning dose of 0.1 mg/kg suspended in 200 μL of cod liver oil. Concentrations of DIM were present in plasma circulation within 15 minutes of administration and gradually decreased 12 hours after administration. The formulation of DIM suspended in liquid oil had the highest bioavailability. The liquid formulation was stable under acidic conditions and provided virtually 100% bioavailability in the animal model. In contrast, crystalline DIM did not show significant bioavailability.[66]

Evidence of the role of DIM in vivo as a chemopreventive agent in animal models of breast cancer substantiates the findings of cell culture experiments. Oral administration of DIM at 5 mg/kg on alternating days was associated with inhibition in the growth of 7,12-dimethylbenzanthracene–induced mammary tumors as well as inhibition of 17β estradiol–induced proliferation of MCF-7 cells in rats.[67] DIM (orally at 5 mg/kg) had an apoptotic effect on 7,12-dimethylbenzanthracene–induced Sprague Dawley rat mammary tumor by negatively regulating the activity of epidermal growth factor receptor and downstream molecules, including Akt.[48] A study in mice has shown evidence that DIM produced a concentration-dependent reduction in the proliferation, migration, invasion, and capillary tube formation of xenograft-transplanted human breast carcinoma.[68] At concentration of 5μM DIM, G_1 cell cycle arrest was demonstrated along with an upregulation in the expression of $p27^{kip}$. The study also showed DIM at a dose of 5 mg/kg inhibited the growth of human MCF-7 cell tumor xenografts by up to 64%. Another study of HER-2/*neu* transgenic mice, DIM has been shown to reduce mammary tumor formation.[69] These data support a role for DIM in inhibiting the invasive capacity of tumor cells, which is a common concern in premenopausal breast cancer[45] and may also play a role in triple-negative disease.[70]

Murine models have also shown evidence of a favorable effect of DIM on estrogen metabolism in relation to breast cancer risk. Sepkovic et al.[71] exposed wild-type mice to standard chow or standard chow plus 0.2% (2000 ppm) DIM for 12 weeks and found enhanced interferon-γ response and lower estradiol concentrations in those fed DIM compared with those fed control chow. It should be noted that the DIM supplementation approximated a 1000-mg single dose in humans. Additionally, the same study showed significantly higher C-2 hydroxylation of estrogen in the DIM-fed mice, reflected by an increased $2OHE_1$:$16\alpha OHE_1$ ratio, further supporting the favorable estrogenic effects of DIM. DIM was more effective than I3C in inducing estradiol-2-hydroxylase in rats, increasing synthesis of $2OHE_1$ in the competing pathways and, consequentially, decreasing $16\alpha OHE_1$.[32] Earlier work has suggested that the $2OHE_1$:$16\alpha OHE_1$ ratio may have prognostic value in breast cancer,[59,72] although the evidence is inconsistent.

EPIDEMIOLOGICAL EVIDENCE

Cruciferous vegetables are the primary source of DIM in the human diet. Data from studies evaluating the association between cruciferous vegetable intake and cancer risk or prognosis are therefore of value in estimating the role of DIM in cancer prevention and control. Table 2 summarizes the epidemiological evidence published since 2000 regarding consumption of cruciferous vegetables and breast cancer risk and recurrence. Overall, point estimates of the odds ratio and relative risk suggest a protective role, though less so in US studies.[1,3,4,9] This may be due to the overall lower intakes in the United States,[11] particularly in comparison with intakes in Asian countries, where the risk of breast cancer is lower among those with greater intakes.[5,7,8] A recent meta-analysis of 13 case–control and prospective cohort studies and 18 673 individual cases suggested that overall high intake of cruciferous vegetables was significantly associated with a 15% lower risk of breast cancer.[2]

With regard to cruciferous vegetable intake and breast cancer recurrence, one study by Thomson et al.[6] suggested that total baseline intake of cruciferous vegetables was associated with a nonsignificant 15% decrease in the hazard rate of recurrence in women with stage I, II, or III invasive breast cancer after an average study duration of 7.3 years. The protective effect reached a significant 35% decrease in women on adjuvant tamoxifen therapy. A pooling of 4 cohorts of women with stages I–III breast cancer by Nechuta et al.[11] showed no significant associations between intake of cruciferous vegetables and breast cancer recurrence or total mortality after a median follow-up of 9 years. In addition to variations in intake across studies, particularly within the highest quartiles, measurement errors in dietary assessment limit the interpretation of the epidemiological evidence.[73]

Table 2 **Epidemiological evidence of cruciferous vegetable intake and breast cancer risk and recurrence since 2000**

Reference	Study population, geographic location (sample size)	Exposure measure	Mean intake (SD) of cruciferous vegetable	OR, RR, or HR (95%CI) for comparison group vs reference group
Studies of breast cancer risk				
Frazier et al. (2003)[3]	Nurses' Health Study, USA (n = 843)	Cabbage, broccoli	Cabbage: 0.14 servings/d Broccoli: 0.07 servings/d	RR: 1.00 (0.64–1.57), Quintile 5 vs Quintile1 RR: 0.74 (0.39–1.41), Quintile 5 vs Quintile 1
Ambrosone et al. (2004)[1]	Erie County and Niagara County hospitals, USA (n = 740)	Broccoli, Brussels sprouts, sauerkraut, coleslaw, cauliflower, cabbage	1531 g/mo (premenopausal) 1368 g/mo (postmenopausal)	Premenopausal Total cruciferous vegetable OR: 0.7 (0.5–1.2), Q4 vs Q1 Broccoli OR: 0.6 (0.4–1.0), Q4 vs Q1 Postmenopausal Total cruciferous vegetable OR: 0.8 (0.6–1.2), Q4 vs. Q1 Broccoli OR: 1.0 (0.7–1.4), Q4 vs. Q1
Adebamowo et al. (2005)[9]	Nurses' Health Study II, USA (n = 90 638)	Broccoli	5–6 servings/wk	RR: 0.99 (0.59–1.65), 5–6 servings/wk vs <1 serving/mo
Zhang et al. (2009)[8]	Hospital-based interviews, China (n = 438 cases/448 controls)	Chinese cabbage, cabbage, broccoli, cauliflower	52.96 (58.23) g/d	Adjusted OR: 0.49 (0.32–0.74), Q4 vs Q1
Boggs et al. (2010)[4]	Black Women's Health Study, USA (n = 51 928)	Broccoli, collard or mustard greens, cabbage, or coleslaw	1–2 servings/wk 3–5 servings/wk ≥6 servings/wk	RR: 0.94 (0.80–1.11), 1–2 servings/wk vs <1 serving/wk RR: 1.01 (0.84–1.21), 3–5 servings/wk vs <1 serving/wk RR: 0.80 (0.65–0.99), ≥6 servings/wk vs <1 serving/wk
Butler et al. (2010)[5]	Singapore Chinese Heath Study, China (n = 34 028)	Total cruciferous vegetables	Q1: 20.2 (14.9) g/1000 kcal Q2: 25.7 (16.3) g/1000 kcal Q3: 30.0 (19.1) g/1000 kcal Q4: 37.1 (24.2) g/1000 kcal	HR, all women: 0.82 (0.63–1.05), Q4 vs Q1 HR, premenopausal women: 1.09 (0.68–1.73), Q4 vs Q1 HR, postmenopausal women: 0.70 (0.51–0.95), Q4 vs Q1
Suzuki et al. (2013)[7]	Japan Public Health Center-based Prospective Study, Japan (n = 47 289)	Cabbage, Japanese radish, Chinese cabbage, komatsuna, broccoli, leaf mustard, qing-geng-cai (bok choy), and chard	Q1: 23 (9.6) g/d Q2: 48 (6.5) g/d Q3: 73 (8.7) g/d Q4: 141 (67.3) g/d	RR, all women: 0.91 (0.7–1.19), Q4 vs Q1 RR, premenopausal women: 0.64 (0.38–1.10), Q4 vs Q1 RR, postmenopausal women: 1.06 (0.78–1.45), Q4 vs Q1
Studies of breast cancer recurrence				
Thomson et al. (2011)[6]	Women's Healthy Eating and Living Study, USA (n = 3088 breast cancer survivors; stage I, II, or III)	Broccoli, broccolini, broccoflower, Bok choy, Brussels sprouts, cauliflower, cabbage, kale, radicchio, mustard/collard/turnip greens, rutabaga, sauerkraut, kohlrabi, watercress, radish, horseradish	0.5 (0.02) servings/d	HR, all women: 0.85 (0.69–1.06), tertile 3 vs tertile 1 HR, tamoxifen-treated women: 0.65 (0.47–0.89), tertile 3 vs tertile 1
Nechuta et al. (2013)[11]	After Breast Cancer Pooling Project, USA/China (n = 18 314 breast cancer survivors; stage I, II, or III)	Total cruciferous vegetables (g/d)	Median: 22.4–25.4 g/d (USA) Median: 93.0 g/d (China)	HR, pooled: 1.10 (0.95–1.28), Q4 vs Q1

Abbreviations: OR, odds ratio; RR, relative risk; HR, hazard ratio; Q, quartile; SD, standard deviation.

MECHANISMS OF ACTION AND SURROGATE ENDPOINTS IN HUMANS

In sum, the current evidence is mixed but compelling enough to validate the initiation of several human intervention studies to further ascertain the cancer-preventive and therapeutic potential of cruciferous vegetables and their bioactive compounds. While few intervention trials have evaluated the role of cruciferous vegetables or DIM in relation to modification of breast cancer risk, several studies have evaluated the effects of cruciferous vegetables or DIM on surrogate breast cancer endpoint biomarkers such as modulation of oxidative stress or metabolism of estrogen.

A cross-sectional analysis of 1005 middle-aged Chinese women found the inflammatory markers tumor necrosis factor α, interleukin 1β, and interleukin 6 to be inversely associated with higher intakes of cruciferous vegetables.[74] Inflammation as well as oxidative stress may contribute to irreparable DNA damage, thereby increasing the risk of cancer. Studies evaluating the role of cruciferous vegetables in modifying oxidative stress include a crossover study by Fowke et al.,[75] who randomized 20 healthy adults to a *Brassica*-rich diet or a vitamin/mineral fiber supplement for 4 weeks, with an intermediate 2-week washout period. The *Brassica*-rich intervention was associated with a 22% reduction in lipid peroxidation as assessed by urinary F_2-isoprostane levels, a stable biomarker of systemic oxidative stress. A larger randomized, placebo-controlled clinical trial[76] of 200 Chinese adults showed that consumption of glucoraphanin-rich beverages (broccoli sprouts) nightly for 2 weeks was not associated with lower aflatoxin-DNA adduct formation but did increase the excretion of these adducts in a subject with high dithiocarbamate levels. In a small trial in 16 healthy adults who consumed 500 g of broccoli per day, the average $2OHE_1$:16αOHE_1 ratio increased, and results indicated that CYP enzymes involved in hydroxylation are induced by dietary broccoli intake.[64]

Studying the physiological responses to the intake of selected bioactive compounds is difficult, due in part to the variations in DIM content of different food sources. On average, 100 g of cruciferous vegetables contains up to 30 mg of glucobrassicin, which is estimated to convert to approximately 2 mg of DIM. However, the variation in DIM content between different cruciferous vegetables is considerable, with differences ranging from 5- to 8-fold.[15] To achieve a biologically relevant exposure, it is suggested that intake would need to be upwards of 600 g/d[77] and sustained for several years for to achieve an anticancer benefit. An intake this high is difficult to attain or maintain through diet alone.

DIM has limited bioavailability because of its extreme insolubility in water and oil. Pure, crystalline DIM is poorly soluble and poorly absorbed upon ingestion. Supplementation with specialized formulations of DIM is necessary to achieve these higher exposures and, in turn, to evaluate the potential chemopreventive activity of DIM in humans. While there are several different DIM formulas available, the majority of monitored and placebo-controlled trials have used BioResponse-DIM (BR-DIM), a dietary supplement containing microencapsulated DIM. Compared with a pure crystalline formulation, BR-DIM is suggested to have 50% higher bioavailability.[26] The half-life of DIM ranges from 2.6 to 4.5 hours, and there is a significant increase in the maximum concentration of plasma DIM at supplementation levels between 100 and 200 mg/d.[78] The recommendation for a tolerable single dose of DIM from BR-DIM has been established as 300 mg (4.3 mg/kg/d).[79] After a single dose of 300 mg of BR-DIM, peak plasma levels of DIM were 236 ng/ml, equivalent to a concentration of 0.94μM DIM.[78] The bioavailability and plasma levels of DIM following oral doses of BR-DIM have been published previously in placebo-controlled human studies.[78–80] Thus far, most clinical research on DIM is based on the consumption of cruciferous vegetables, but current clinical trials are examining the effects of DIM, specifically the BR-DIM formulation, on breast cancer risk.

The number of intervention studies with DIM supplementation remains limited. In a study of *BRCA1*-inherited mutation, a mutation associated with a high lifetime risk of breast cancer,[81] BR-DIM providing DIM at 300 mg/d (150 mg twice daily) showed a borderline significant ($P = 0.05$) increase in *BRCA1* expression in healthy women aged 25 to 63 years. In the 13 women with the inherited *BRCA1* mutation, 4 to 6 weeks of DIM supplementation resulted in an average 34% (range −24% to 194%) increase in *BRCA1* mRNA expression.[82] In a study of 20 healthy women with a *BRCA1* mutation, Nikitina et al.[83] evaluated the effect of 4 to 6 weeks of BR-DIM supplementation on the urinary $2OHE_1$:16αOHE_1 ratio. This short-term intervention showed no significant change in the $2OHE_1$:16αOHE_1 ratio ($P = 0.35$), regardless of menopausal status.

A placebo-controlled, double-blind trial on BR-DIM was conducted in 19 postmenopausal women with early-stage breast cancer. Women were randomized to receive 108 mg of BR-DIM or placebo daily for 30 days. When compared with placebo, BR-DIM resulted in a significant increase in $2OHE_1$ ($P = 0.02$) and a modestly protective shift in the $2OHE_1$:16αOHE_1 ratio ($P = 0.059$).[84] The ability of DIM to induce CYP enzymes responsible for catalyzing hydroxylation could be responsible for the enhanced production of $2OHE_1$ and the limited production of 16αOHE_1. Additionally, administration of BR-DIM to premenopausal women

Table 3 **Completed and ongoing clinical trials testing the efficacy of diindolylmethane in breast cancer prevention**[a]

Study title	Clinicaltrial.gov identifier	Principal investigator	Purpose	Intervention	Date of study completion	Publication
Phase I ascending single dose pharmacokinetics and safety study of 3,3′ diindolylmethane	NCT00784394	A. Hurwitz	Study the side effects and best dose of DIM in preventing cancer in healthy volunteers	Single-dose DIM (BR-DIM); response observed over 24 h	October 2009	Reed et al. (2008)[79]
Phase 1 multiple-dose safety, pharmacokinetic, and drug interaction clinical study of nutritional-grade, absorption-enhanced DIM (BR-DIM)	NCT00392652	G. Reed	Study the side effects and best dose of DIM in healthy volunteers	Low- and higher-dose BR-DIM twice daily for 4 wk	October 2009	N/A
The potential for oral DIM supplementation to increase the production of the BRCA1 protein in *BRCA1* mutation carriers	NCT01022333	S.A. Narod	Determine whether there is potential for oral DIM supplementation to result in increased production of BRCA1 protein in *BRCA1* mutation carriers	300 mg of DIM (BR-DIM) daily for 6 wk	July 2010	Nikitina et al. (2015)[83]
Effect of cruciferous vegetables or cruciferous supplement on urinary estrogen metabolites in premenopausal women	NCT01726127	S.A. Tanumihardjo	Evaluate ratio of urinary estrogen metabolites in healthy premenopausal women	Consumption of 40 g of broccoli or Brussels sprouts daily for 8 wk, followed by Cruciferous Complete supplement for 8 wk	July 2015	N/A
Evaluation of diindolylmethane supplementation to modulate tamoxifen efficacy in breast cancer: the diindolylmethane efficacy study	NCT01391689	C. Thomson	Phase II/III trial to study how well DIM works and to compare DIM with placebo in treating patients with breast cancer	DIM (BR-DIM) orally twice daily for ≈36 mo	September 2016 (estimated)	N/A
A nutritional intervention to decrease breast density among female BRCA carriers – a prospective clinical trial	NCT02197000	D. Margel	Determine whether adding DIM supplement will decrease breast density among female BRCA mutation carriers over a period of 2 y	100 mg of DIM-Avail (nutritional supplement) once daily for 24 mo	January 2018 (estimated)	N/A

Abbreviations: BR-DIM, BioResponse diindolylmethane; DIM, diindolylmethane; Q, quartiles; N/A, not available.
[a]Information obtained from clinicaltrials.gov on February 4, 2016.

improves symptoms of cyclical mastalgia,[85] a potential indicator of increased breast cancer risk.[86] To date, many trials evaluating DIM and breast cancer have been conducted in samples of less than 50 subjects, limiting the interpretation of study findings. Several trials evaluating the role of DIM in breast cancer prevention are currently under way (Table 3).

CONCLUSION

DIM and its precursor I3C are among the most commonly evaluated indoles found in cruciferous vegetables. These compounds have been widely studied in relation to breast cancer chemoprevention.[87] Numerous mechanisms by which dietary exposure to these compounds may modulate breast cancer have been reported, including apoptosis, modulation of response to oxidative stress, estrogen metabolism, and cell cycle modulation, and other antiproliferative activities have been evaluated, largely in cell culture and animal studies.[24,30] The evidence for a protective role of DIM against breast cancer continues to grow, but additional research is needed to further identify and refine the mechanistic targets of this compound, particularly in humans. DIM is available to consumers in a generic crystalline formulation (low bioavailability) and in a microencapsulated form as BR-DIM (higher bioavailability). Patient inquiries regarding the possible use of DIM as protective or adjuvant therapy during chemotherapy are mounting, in part because of the increasing availability of and information on DIM. Nevertheless, information about the specific dosing of DIM and the corresponding intakes of cruciferous vegetables is currently lacking. Before any recommendations can be developed, clinical trials must be completed to determine the evidence-driven basis for a dietary recommendation.

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