Chapter 6
Indole-3-Carbinol and Its Role in Chronic Diseases

Barbara Licznerska and Wanda Baer-Dubowska

Abstract Indole-3-carbinol (I3C), a common phytochemical in cruciferous vegetables, and its condensation product, 3,3'-diindolylmethane (DIM) exert several biological activities on cellular and molecular levels, which contribute to their well-recognized chemoprevention potential. Initially, these compounds were classified as blocking agents that increase drug-metabolizing enzyme activity. Now it is widely accepted that I3C and DIM affect multiple signaling pathways and target molecules controlling cell division, apoptosis, or angiogenesis deregulated in cancer cells. Although most of the current data support the role of I3C and DIM in prevention of hormone-dependent cancers, it seems that their application in prevention of the other cancer as well as cardiovascular disease, obesity, and diabetes reduction is also possible. This chapter summarizes the current experimental data on the I3C and DIM activity and the results of clinical studies indicating their role in prevention of chronic diseases.

Keywords Indole-3-carbinol · DIM · Signaling pathways · Chronic diseases · Animal models · Dietary intervention trials

6.1 Introduction

The plant family *Cruciferae*, particularly members of the genus *Brassica*, like cabbage, broccoli, cauliflower, Brussels sprouts, kale, bok choy are rich sources of sulfur-containing glucosinolates. These secondary products of plant metabolism include, among others, glucobrassicin and neoglucobrassicin. When plant tissue is disrupted, an endogenous thioglucosidase (myrosinase) is activated and converts

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glucobrassicin and other indolylic glucosinolates to indoles, principally to indole-3-carbinol (I3C) [120].

Since the first reports on possible anti-carcinogenic activity of I3C [122] numerous preclinical studies have confirmed the chemopreventive properties of this compound by preventing, inhibiting, and reversing the progression of cancer. Moreover, preliminary clinical trials have shown that I3C is a promising agent protecting against hormone-dependent as well as hormone-independent human cancers [47].

Thus, it is not surprising that there are many marked diet supplements containing I3C. Several mechanistic studies have been performed in order to elucidate the mechanism of pleiotropic activity of I3C.

This chapter summarizes the current knowledge on the possible interference of I3C with signaling pathways in vitro and in animal models, as well as its application in prevention of chronic diseases.

6.2 Physicochemical Properties and Pharmacokinetics of Indole-3-Carbinol

Among the indoles, generated upon ingestion of cruciferous vegetables, only I3C (IUPAC: 1H-indol-3-ylmethanol) is commercially available as an off-white solid. Basic physical and chemical properties of I3C are summarized in Table 6.1.

I3C is chemically unstable in acidic conditions, in vitro in cell cultures and in vivo in the stomach environment. In such conditions, I3C may rapidly condense into a series of oligomeric products, of which a dimer, 3,3,-diindolylmethane (DIM), is considered the most bioactive product (Fig. 6.1) [2, 115]. Several pharmacokinetics studies, performed mostly in animal models, have been conducted for I3C and its condensation products [5, 6, 34, 39, 105]. When rainbow trout has been administered with radiolabeled [5-^{3}H]-indole-3-carbinol, 40 % of total radioactivity was found in the liver extracts as DIM [34]. Upon oral administration of 250 mg/kg to mice, the I3C was rapidly absorbed and distributed into variety of tissues and body fluids (e.g., liver, kidney, lung, heart, brain, and plasma) with highest concentrations in liver and kidney, but with rapid clearance (concentrations below the limit of detection within 1 hour after administration). In the same experiment, DIM was detected in plasma at 15 min and was still quantifiable after 6 h with a peak at 2 h after I3C dosing [5, 6]. DIM was also found in stomach tissue and contents,

Table 6.1 Physical and chemical properties of I3C (ALOGPS, www.pubchem.com; accessed Dec 26, 2015)

Stability	Off-white powder
Molecular weight	147.17386 g/mol
Melting range	96–99 °C
Storage temperature	2–8 °C
Stability	2–80 °C, considered stable
Water solubility	3.75 mg/ml, mixes with water

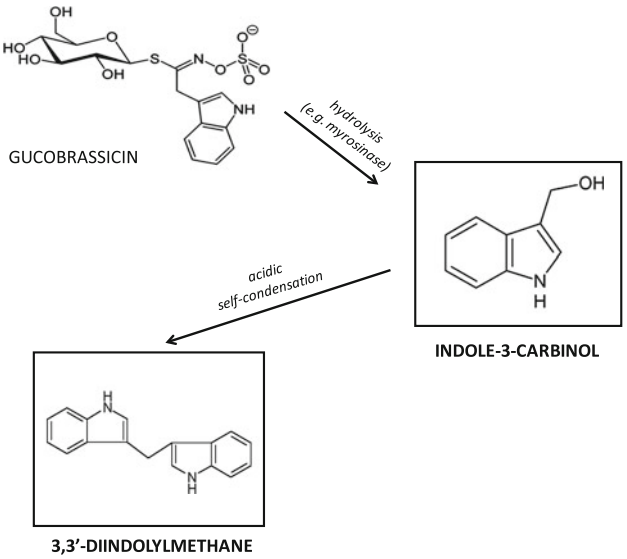

Fig. 6.1 Molecular structure and formation of I3C and DIM

intestines, and liver after 1 h following oral administration of I3C to rats [39]. In human volunteers intervention trial DIM was detected in plasma within 8 h following of 400 mg I3C oral dose [9]. In a phase I clinical trial in women, no I3C was found in the plasma after administration of a single dose of up to 1200 mg or multiple-doses at 400 mg provided twice daily for 4 weeks, and DIM was the only detectable I3C-derived compound in plasma [105]. Fujioka et al. [47] have found that urinary DIM level after uptake of I3C from Brussels sprouts or cabbage is a biomarker of glucobrassicin exposure in humans. All these results support the suggestion that I3C serves as the prodrug rather than the therapeutic agent itself. In this regard, purified I3C as treatment agent used in in vitro models seems to be somewhat contradictory, because there is no certainty that any metabolism of DIM in cells occur. Thus, in this chapter the biological activity and the role in chronic diseases will refer not only to I3C but also to DIM, its major condensation product in humans.

6.3 Modulation of Cell Signaling Pathways by Indole-3-Carbinol

I3C affects multiple signaling pathways and target molecules controlling cell division, apoptosis or angiogenesis deregulated in cancer cells. Figure 6.2 presents the overview of the signaling pathways and possible crosstalks influenced by I3C or DIM. One of the major pathways targeted by I3C is phosphoinositide 3-kinase

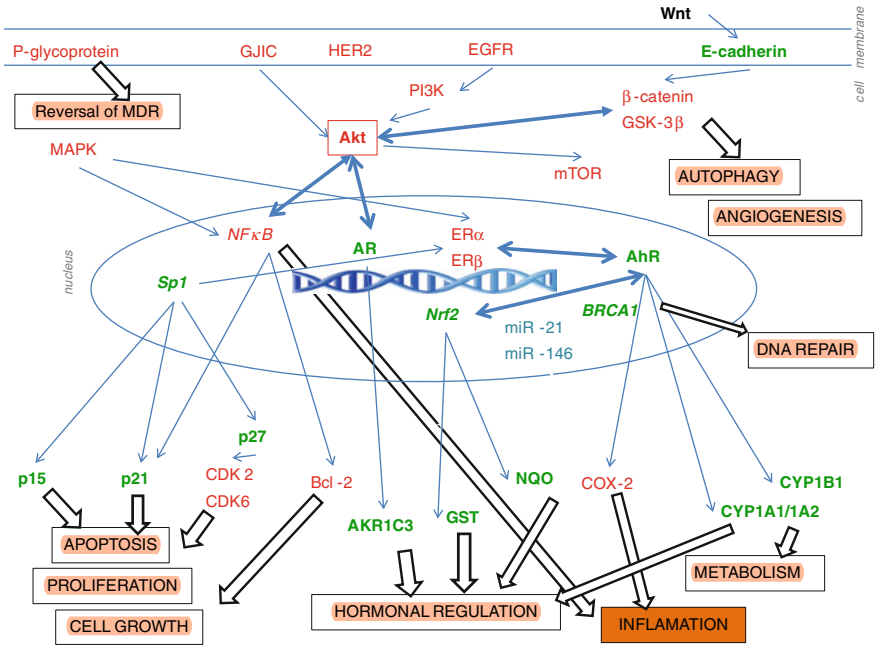

Fig. 6.2 Signaling pathways and proposed crosstalks (↔) affected by I3C/DIM; *red*-inhibition, *green*-induction (AhR-aryl hydrocarbon receptor, AKR-aldo-keto reductase, Akt- protein kinase B, AR-androgen receptor, BRCA-breast cancer tumor suppressor gene, CDK-cyclin-dependent kinase, COX-cyclooxygenase, EGFR-epidermal growth factor receptor, ER-estrogen receptor, GJIC-Gap junctional intracellular communication, GSK-glycogen synthase kinase, GST-glutathione-S-transferases, HER-receptor tyrosine-protein kinase erbB-2, MAPK-mitogen-activated protein kinases, MDR-multidrug resistance, NFκB-nuclear factor kappa-light-chain-enhancer of activated B cells, Nrf2-nuclear transcription factor 2, NQO-NAD (P)H:quinone oxidoreductases, PI3K-phosphoinositide 3-kinase, Sp1-Sp1 transcription factor)

(PI3K)—protein kinase B (Akt)—mammalian target of rapamycin (mTOR) signaling pathway. This pathway is a cascade of events that plays a key role in the broad variety of physiological and pathological processes. PI3K/Akt/mTOR signaling pathway is one of the most frequently affected target in all sporadic human cancers, and it has been estimated that mutations in the individual component of this pathway account for as much as 30 % of all known human cancers [83, 112].

Akt is a serine/threonine protein kinase functioning downstream of PI3K in response to mitogen or growth factor stimulation. The inhibition of phosphorylation and subsequent activation of Akt kinase by I3C or DIM was described in prostate and breast cancer cells. In addition, I3C abrogated epidermal growth factor (EGF)-induced activation of Akt in prostate cancer cells. Furthermore, the known downstream modulators of the Akt/PI3K cell survival pathway, Bcl-x(L), and BAD proteins showed decreased expression after I3C treatment [24, 49]. Several genes that mediate processes involved in carcinogenesis are regulated by transcription

factor NF-κB. It plays a central role in general inflammation as well as immune responses, although recent evidences suggest that its role in these processes is more complex [73]. The enhanced activation and expression of NF-κB is linked to development and progression of human cancers as well as in the acquisition of drug-resistant phenotype in highly aggressive malignancies [3, 109].

Activation through Akt is important for many tumorigenic functions of NF-κB. Several studies showed that both indoles inhibited PI3K/Akt/mTOR/NF-κB signaling (reviewed in [4]).

It was also known that there is a crosstalk between Akt and Wnt signaling pathways through the signal communication between glycogen synthase kinase 3 (GSK-3β) and β-catenin, two of the important molecules in Akt and Wnt pathways, respectively [109]. It was found that DIM significantly increased the phosphorylation of β-catenin, and inhibited β-catenin nuclear translocation [77] suggesting that DIM could also downregulate the activation of Wnt signaling.

Mitogen-activated protein kinases (MAPK) are involved in cellular response to a diverse array of stimuli and regulate several cell functions including differentiation, mitosis, cell survival, and apoptosis [100]. Downregulation of the expression of MAP2K3, MAP2K4, MAP4K3, and MAPK3 by I3C and DIM was described suggesting their inhibitory effects on MAPK pathway [76]. It was also reported that the effects of DIM were mediated by crosstalk between protein kinase A and MAPK signaling pathways [75].

Gap junctional intracellular communication (GJIC) also involved in regulating cell proliferation, differentiation and apoptosis is modulated by gap junction channels via broad variety of endogenous and exogenous agents [90]. I3C was reported to prevent H_2O_2-induced inhibition of GJIC by the inactivation of Akt in rat liver epithelial cells [57].

Several studies have focused on the potential effects of I3C and DIM on the proliferation and induction of apoptosis in human prostate or breast cancer cell lines. Cell G_1 cycle arrest of breast cancer cells by I3C was related to inhibition of the expression of cyclin-dependent kinase-6 (CDK6) independently of estrogen receptor signaling [30]. Moreover, the inhibition of CDK6 expression in human MCF7 breast cancer cells was further explained by disrupting Sp1 transcription factor interaction with CDK6 gene promoter [32].

In prostate cancer cell lines, the induction of apoptosis by I3C was p53-independent [94]. The induction of $p21^{WAF1}$ expression by DIM was also independent of p53 status [55]. Pro-apoptotic effect of DIM in HER2/Neu overexpressing breast cancer cells was connected to inhibition of HER2/Neu activity [87]. We have also found a decreased expression of HER2/Neu in estrogen independent MDA-MB-231 breast cancer cells treated with either I3C or DIM (Licznerska et al. unpublished data).

I3C has been known to be a negative regulator of estrogens, while DIM a negative regulator of androgens. I3C inhibited the transcriptional activity of ERα, the estradiol-activated ERα signaling, and the expression of the estrogen-responsive genes [89]. Since I3C and DIM could also inhibit the proliferation of ER-negative breast cancer cells, it is suggested that antitumor activities of indoles could be ER

independent. DIM was found to be an antagonist of AR, inhibiting androgen-induced AR translocation to the nucleus [74]. DIM also enhanced expression of aldo-keto reductase 1C3 (AKR1C3), an enzyme responsible for inactivation of 5α-dihydrotestosterone and decreasing estrogen levels in mammary gland and eliminating active androgens from the prostate [92, 101].

Several lines of evidence indicate possible crosstalk between ERα and AhR signaling pathways [96]. It was shown that I3C triggers AhR-dependent ERα protein degradation in MCF7 breast cancer cell line disrupting an ERα-GATA3 transcription factor cross-regulatory loop. This lead to ablation of ERα expression and loss of ERα-responsive proliferation [86].

Our studies showed that both indoles upregulated AhR and downregulated ERα expression in non-tumorigenic MCF10A and tumorigenic MCF7 breast epithelial cells [114]. Upregulation of AhR was also observed in ER-negative MDA-MB-231 cells. This observation is important since increased expression and activation of AhR result in induction of CYPs involved in estrogen metabolism [113].

Both I3C and DIM elicited an inhibition of cell adhesion, migration and invasion in the breast cell lines of different ER status [89]. This appeared to be due, in part, to I3C-induced upregulation of the tumor suppressor gene *BRCA1* and other proteins involved in DNA repair, like RAD51 [105].

Recent studies show that AhR and ERα interact with and modulate the activity of Nrf2 [52]. This transcription factor plays an essential role in cellular protection against electrophiles and oxidative stress by upregulating expression of phase II detoxifying enzymes, including glutathione-S-transferases (GST) and NAD(P)H: quinone oxidoreductases (NQO) [81]. Significant increase of the Nrf2 transcript level in MCF7 and MDA-MB-231 breast cancer cell lines was observed after treatment with I3C. Moreover, increase of Nrf2 expression was correlated with enhanced expression of NQO1 or GSTP in MCF7 cells and MDA-MB-231 cells respectively [114].

Recently, a plethora of evidence has demonstrated that epigenetic alterations, such as DNA methylation, histone modifications, and non-coding miRNAs, consistently contribute to carcinogenesis, and dietary phytochemicals, including glucosinolate derivatives, have the potential to alter a number of these epigenetic events [46, 119].

In this regard, DIM was reported to decrease promoter methylation of Nrf2 in vitro in TRAMP C1 mouse prostate cell line and in vivo in TRAMP mice prostate tumors. This effect was at least in part related to decreased expression and activity of DNA methyltransferases [130]. Moreover, genome-wide promoter methylation in normal and cancer prostate cells showed broad and complex effects on DNA methylation profiles reversing many of the cancer associated methylation alterations, including aberrantly methylated genes that are dysregulated or are highly involved in cancer progression [129].

Histone modifications by DIM were found in several studies. Decreased histone deacetylases (HDACs) protein expression were observed in human colon, breast,

and prostate cancer cell lines, human colon cancer xenografts in nude mice and in mouse prostate cells, influencing apoptotic proteins, like p21, p27, and involved in inflammation COX-2 [46].

I3C and DIM were found to downregulate miR-21 and miRNA-146, respectively, in Panc-1 pancreatic cancer cells, which was related to induction of chemosensitivity to gemcitabine in these cells [78, 98]. In another study, I3C reversed the upregulation of miR-21 caused by lung carcinogen—vinyl carbamate in mice [88]. DIM upregulated miR-21 in breast cancer MCF7 cells resulting in reduced proliferation [62], and the let-7 family miRNA leading to inhibition of self-renewal and clonogenic capacity of prostate cancer cells [69, 70].

To sum up, both I3C and its dimer demonstrate pleiotropic effects on cell signaling and subsequently gene expression regulation. Some of these activities are summarized in Table 6.2.

Table 6.2 Summary of the major biological effects of indole-3-carbinol (I3C) and its condensation product—3,3'-diindolylmethane (DIM)

Effect[a]	Target molecules	I3C		DIM	
		In vitro	In vivo	In vitro	In vivo
Induction of phase I enzymes	CYP1A1, CYP1A2, CYP1B1	+	+	+	
Induction of phase II enzymes	GST, NQO	+	+	+	+
Inhibition of DNA adducts formation	CYP1B1		+		
Anti-estrogenic activity	ER, AhR	+	+	+	
Anti-androgenic activity	AR	+		+	
Cell cycle arrest	p21, p27, CDK6	+		+	
Pro-apoptotic	Akt, NFκB, GSK3β, JNK	+		+	
Anti-angiogenic activity	E-cadherin, α-, β-, and γ-catenins, MMP9	+		+	+
Anti-proliferative activity	ERβ/ERα	+	+	+	
DNA repair	BRCA1, RAD51	+	+	+	
Reversal of MDR	P-glycoprotein	+	+		
Epigenetic modifications	DNMTs, HDACs, miRNAs			+	+
Anti-inflammatory activity	NFκB, COX-2	+	+	+	

AhR aryl hydrocarbon receptor, *Akt* protein kinase B, *AR* androgen receptor, *BRCA* breast cancer tumor suppressor gene, *CDK* cyclin-dependent kinase, *DNMT* DNA methyltransferase, ER-estrogen receptor, *GSK* glycogen synthase kinase, *GST* glutathione-S-transferases, *HDAC* histone deacetylases, *JNK* c-Jun N-terminal kinase, *MDR* multidrug resistance, *MMP* matrix metalloproteinase, *NFκB* nuclear factor kappa-light-chain-enhancer of activated B cells, *NQO* NAD(P)H:quinone oxidoreductases

[a]References in the text

6.4 Role of Indole-3-Carbinol in Chronic Diseases

The term "chronic diseases" appear under different names in different contexts. According to WHO this term suggests the following shared features:

- The chronic disease epidemics take decades to become fully established—they have their origins at young age;
- Given their long duration, there are many opportunities for prevention;
- They require a long-term and systematic approach to treatment (WHO Report 2015).

In this context, cardiovascular diseases, chronic respiratory diseases, diabetes along with cancer are mentioned. However, chronic character of cancer is not so obvious as in the case of the other illnesses. The idea of considering cancer as chronic disease emerged recently when it was noted that many cancers, while still very serious could be manageable chronic diseases with ongoing surveillance and/or treatment. While this vision has not yet become a reality for most forms of cancer, the past 10–20 years have brought about a marked acceleration in advance toward this goal. In this regard some types of metastatic breast cancer have become manageable over the long term, perhaps most famously with tamoxifen, which can slow or stop malignant cell growth in many women with estrogen-dependent cancer by blocking hormone receptors on tumor cells. Moreover, a new class of aromatase (*CYP19*) inhibitors that target estrogen production was developed, which seem to provide better results than tamoxifen [127]. I3C can also inhibit the expression of aromatase as well as CYP isoforms involved in estrogen metabolism. Therefore, I3C, as well as its condensation product DIM, appear to be a promising agent for the prevention or recurrence of human tumors, particularly hormone-dependent cancers. There are also data suggesting that I3C or DIM might also be useful for prevention or recurrence of cardiovascular diseases, diabetes, or recurrent respiratory papillomatosis, as well as obesity.

6.4.1 *The Role of I3C in Cancer Controlling*

It is almost 40 years after Lee Wattenberg and William Loub [122] for the first time reported that I3C inhibited chemically induced breast and forestomach neoplasia in rodents. Since then the antitumor activity of dietary I3C has been widely studied and the inhibition of the development of other cancer types, including liver [95], lung [63], and prostate [110] have been demonstrated.

Breast cancer is the most common and the leading cause of cancer mortality among women worldwide. The preventive efficacy of I3C on mammary carcinoma first observed in animal models, was confirmed in many mechanistic studies in cell cultures and was supported by epidemiological studies with cruciferous vegetables and their extracts or juices [117, 120].

One of the most important risk factor of breast tumors are estrogens, which are classified as carcinogenic in humans [58]. These steroid hormones may contribute to breast cancer development in two ways: (i) acting as promoters by stimulating cell proliferation, (ii) inducing genotoxicity through the reaction of their active metabolites with DNA thus acting as tumor initiators.

Experimental studies in vitro in breast epithelial cell lines showed that I3C, DIM, and cabbage juices induce CYP450 genes *CYP1A1, 1A2, 1B1* encoding the key enzymes of estrogen catabolism. The profile of metabolites was in favor to 2-hydroxyestrogens being noncarcinogenic in comparison to estradiol and 4-hydroxy derivatives [80, 114]. This anti-estrogenic activity of I3C could be explained by the induction of AhR receptor showed in other studies [114].

Moreover, I3C, DIM, and cabbage juices are capable of upregulating phase II detoxifying enzymes, including GST and NQO in breast cancer cell lines [114, 120]. Upregulation of GSTs and NQO1 by I3C was correlated with increased levels of Nrf2, in benign MCF7 and aggressive MDA-MB-231 breast cancer cell lines. Thus, it may be assumed that I3C protects against estrogen-associated carcinogenesis by removal of the genotoxic metabolites of estrogens.

Simultaneously, I3C and DIM influenced in situ production of estrogens in breast epithelial MCF7 cancer cells by reducing the expression of aromatase (*CYP19*), the enzyme that synthesizes estrogens by converting C19 androgens into aromatic C18 estrogenic steroids [80]. Several studies have shown that there is an overexpression of aromatase gene in breast cancer tissue [82]. Interestingly, the potential of I3C to reduce estrogenic activity in the breast cancer cells was confirmed by other mechanisms, particularly via decreasing the AKR1C3 expression mentioned in the previous section. In the mammary gland where enzyme converts androstenedione to testosterone—one of the aromatase substrates [92, 101].

Besides the interference with estrogens metabolism pathways, I3C also affects DNA repair, the cell cycle progression and apoptosis in breast cancer cell lines. In this regard it was shown that I3C induces BRCA1 expression and that both I3C and BRCA1 inhibited estrogen (E2)-stimulated ERα activity in human breast cancer cells [44]. BRCA1 is DNA repair factor involved in repair of DNA double-strand breaks. *BRCA1*gene expression is reduced or completely silenced in a significant proportion of sporadic breast cancer because of hypermethylation of the gene promoter [106, 126]. Thus, I3C-induced BRCA1 expression and inhibition of estrogen-stimulated ERα activity by I3C and BRCA1 showed by some studies could be one of the antitumor activity of the indole [44].

Several lines of evidence suggest I3C ability to arrest cell cycle in breast cancer cells. In this regard I3C was reported to inhibit CDK2 activity in breast cancer MCF7 cell line [48]. Moreover, both I3C and DIM upregulated CDK inhibitors p21 and p27, although in a very high concentration of 200 μM. Activity of this protein is especially critical during the G_1 to S phase transition. Consequently, the ability to arrest G_1 phase by I3C was shown [30]. Other studies suggest the p53 phosphorylation by I3C leading to release p53 and inducing the p21 CDK inhibition and G_1 cell cycle arrest [18, 86]. Importantly, treatment with I3C and tamoxifen ablated expression of the phosphorylated retinoblastoma protein (Rb), an endogenous

substrate for the G_1 CDKs, whereas either agent alone only partially inhibited endogenous Rb phosphorylation [31]. Several studies showed that I3C and its derivatives are potent inducers of apoptosis in both ER-positive and ER-negative breast cancer cells[30–32, 50, 80, 103]. Estrogens, particularly estradiol have been also implicated as a cofactors in human papillomavirus (HPV)-mediated cervical cancer, both in animal models and in women using oral contraceptives [72]. Interestingly, it was found that estradiol protects cervical cancer cells treated with DNA-damaging agents such as UVB, mitomycin-C, and cisplatin, from apoptotic death. I3C was able to overcome the anti-apoptotic effect of estradiol but only in higher concentrations. Treatment with I3C resulted in loss of the survival protein Bcl-2. However, the amount of apoptosis versus survival and the level of Bcl-2 depended on the I3C/estradiol ratio [22]. In HPV16-transgenic mice, which develop cervical cancer after chronic estradiol exposure, apoptotic cells were detected in cervical epitheliumonly in mice exposed to estradiol and fed on I3C [20].

Experiments in which cervical cancer HeLa and SiHa cell lines were used, demonstrated that DIM also exerts antitumor effects on these cells through its anti-proliferative and pro-apoptotic roles, especially for SiHa cells. The molecular mechanism for these effects may be related to its regulatory effects on MAPK and PI3K pathway and apoptosis proteins. Thus, DIM may be considered a preventive and therapeutic agent against cervical cancer [135]. The ability of inhibiting spontaneous occurrence of endometrial adenocarcinoma and preneoplastic lesions by I3C was also demonstrated in female Donryu rats. It was suggested that this effect was due to induction by I3C estradiol 2-hydroxylation [68]. On the other hand, promotion of endometrial adenocarcinoma in same strains of rats initiated with N-ethyl-N'-nitro-N-nitrosoguanidine by I3C was described [133]. DIM was also found to have a potent cytostatic effect in cultured human Ishikawa endometrial cancer cells. This effect was related to the stimulation of TGF-α expression and activation of TGF-α signal transduction pathway [75].

Another hormone-dependent cancer, which might be affected by I3C, is prostate cancer, one the most prevalent malignancy in men worldwide and the second leading cause of male death in Western countries [16]. Androgens play a critical role in prostate cancer cells growth and survival. Androgens bind to the androgen receptor (AR), a steroid nuclear receptor, which is translocated into the nucleus and binds to AREs in the promoter regions of target genes to induce cell proliferation and apoptosis. Approximately 80–90 % of prostate cancers are dependent on androgen at initial diagnosis, and endocrine therapy of prostate cancer is directed toward the reduction of plasma androgens and inhibition of AR [37, 54]. It was demonstrated that both I3C and DIM are able to downregulate AR signaling [74], but only DIM was shown to be a strong antagonist of AR and inhibitor of its translocation to the nucleus [30, 74].

Similarly as in the case of breast cancer cells, I3C and its derivatives also affect cell cycle progression and induce apoptosis. In this regard, cell cycle arrest at G_1 checkpoint in different human prostate carcinoma cell lines by I3C and DIM was described [2]. In LNCaP prostate cancer cells I3C selectively inhibited the expression of CDK6 protein and transcripts and stimulated the production of the

p16 CDK inhibitor. In vitro protein kinase assays revealed inhibition by I3C CDK2 enzymatic activity and the relatively minor downregulation of CDK4 enzymatic activity [134].

In PC-3 cell line induction of G_1 cell cycle arrest by I3C due to the upregulation of p21(WAF1) and p27(Kip1) CDK inhibitors, followed by their association with cyclin D1 and E and downregulation of CDK6 protein kinase levels and activity was suggested. In addition, I3C inhibited the hyperphosphorylation of the retinoblastoma (Rb) protein in PC-3 cells. Induction of apoptosis was also observed in this cell line when treated with I3C. Thus, it was suggested that I3C inhibits the growth of PC-3 prostate cancer cells by inducing G_1 cell cycle arrest leading to apoptosis, and regulates the expression of apoptosis-related genes [23]. Further studies showed that I3C-induced apoptosis is partly mediated by the inhibition of Akt activation, resulting in the alterations in the downstream regulatory molecules of Akt activation in PC-3 cells [24]. In the case of DIM an inhibition of a crosstalk between Akt and NF-κB [12], leading to cell cycle arrest and induction of apoptosis was also described. DIM significantly decreased cellular histone deacetylase HDAC2 protein level in androgen sensitive LNCaP and androgen insensitive PC-3 cell lines [10]. In all these studies a formulated DIM (BR-DIM) with higher bioavailability was used and was able to induce apoptosis and inhibit cell growth, angiogenesis, and invasion of prostate cancer cells [21].

The potential protective activity of I3C and DIM against prostate cancer was confirmed by microarray analysis, which showed the modulation of the expression of many genes related to the control of carcinogenesis and cell survival as effect of indoles treatment of PC-3 cells [76]. It was also demonstrated by several groups that I3C and DIM may improve the therapeutic effect of conventional chemotherapy of prostate cancer [44, 71].

Besides the hormone-dependent cancers, both indoles can affect the development of some other cancers. In this regard, it was shown that I3C and DIM induced apoptosis in colorectal cancer cell lines [13, 67, 84]. Interestingly, an effective inhibition of Akt and inactivation of mTOR was observed as a result of combined treatment with I3C and genisteinin HT29 colon cancer cells, leading to induction of apoptosis and autophagy [93].

Anti-carcinogenic activity of I3C was demonstrated in carcinogen-induced lung cancer in mice [63, 64]. Anti-proliferative effects of I3C and DIM in human bronchial epithelial cells (HBEC) and A549 adenocarcinomic human alveolar basal epithelial cells related to marked reductions in the activation of Akt, extracellular signal-regulated kinase and NF-κB were also described [65].

Moreover, upregulation of several miRNAs induced by chemical carcinogen was reversed by I3C in mice and rats lung tumors [46, 59].

The signal transducer and activator of transcription 3 (STAT3) is a latent transcription factor required in proliferation and differentiation. The constitutive activation of STAT3 in human pancreatic carcinoma specimens but not in normal tissues was shown. Activation of STAT3 was also found in pancreatic tumor cell lines and was inhibited by I3C although in relatively high concentration (10 μM) along with induction of apoptosis [79]. Apoptosis in pancreatic cancer cells was

also induced by DIM as a result of endoplasmic reticulum stress-dependent upregulation of death receptor 5 [1]. More recent studies showed downregulation of miRNA-21 and miRNA-221 as a result of I3C or DIM treatment of pancreatic cancer cells. As upregulation of these miRNAs is characteristic for more aggressive pancreas cancer, it was suggested that combination of I3C or DIM with conventional chemotherapeutics may increase the chemosensitivity to certain drugs in resistant pancreatic cancer cells [98, 111].

There are also reports showing the anti-proliferative effect, G1 cell cycle and induction of apoptosis in thyroid cancer cells by I3C and DIM [116]. However, earlier reports indicated the enhancement of thyroid gland neoplastic development by I3C in rat medium-term multi-organ carcinogenesis model [66]. UVB-induced mouse skin tumors were reduced in mice fed on I3C [29].

Importantly it was also shown that I3C may overcome multiple drug resistance by downregulation of MDR1-expression in murine melanoma cells and leukemia cells [7, 8, 28].

These observations further support the possible application of I3C or DIM as potential adjuvant therapeutics in conventional chemotherapy of several cancers.

Finally, it must be pointed out that although I3C was shown to have anti-carcinogenic activity in various animal models, at the same time animals studies have also shown a tumor-promoting activity, when animals were exposed to I3C after exposure to carcinogens [131, 133]. This aspect has to be clarified in long-term studies.

6.4.2 *Cardiovascular Diseases*

Cardiovascular diseases still remain the primary cause of death worldwide. One of the proposed approaches to reduce the high global incidence is the consumption of vegetables and fruits containing biologically active components or phytochemical supplements. Although the most attention was paid to resveratrol, components of cruciferous vegetables, particularly *Brassica oleracea*, were also considered a potential dietary phytochemicals reducing risk of CVDs [97].

In this regard hypocholesterolemic properties of I3C were reported in mice provided with cholesterol-supplemented diet to which I3C were added. Since in vitro experiments revealed that I3C and its condensation products effectively inhibited the enzyme acyl-CoA:cholesterol acyltransferase (ACAT), which is responsible for the conversion of free cholesterol to the cholesteryl ester, the hypocholesterolemic effect of I3C in mice was likely mediated by the inhibition of ACAT [42]. Such mechanism was further confirmed in HepG2 cells. As a result of treatment with I3C the decreased cholesteryl ester synthesis was associated with significantly decreased ACAT gene expression and activity [85].

Moreover, antiplatelet and antithrombotic activity of I3C was shown in in vitro and in vivo studies. I3C significantly inhibited collagen-induced platelet aggregation in human platelet-rich plasma and suppressed the death of mice with

pulmonary thrombosis induced by intravenous injection of collagen and epinephrine [99].

The protective activity of I3C in heart failure and vascular proliferative disease was also reported. In this regard, it was shown that I3C can suppress the proliferation of cultured vascular smooth muscle cells and neointima formation in a carotid injury model via the Akt/GSK3β pathway [51]. Vascular smooth muscle cells are the principal cell types involved in the pathogenesis of atheriosclerosis and restenosis after percutaneous coronary intervention [43]. Thus, it was suggested that I3C may be a part of new therapeutic strategy for vascular proliferative diseases as well as heart failure. The latter suggestion was supported by the results of the studies using aortic banding (AB) mouse model, which showed that I3C prevented and reversed cardiac remodeling induced by AB. This effect was mediated by AMPK-α and extracelular signal-regulated kinases 1/2 (ERK1/2) [36]. Since AMPK acts as important energy sensor, attenuation of cardiac remodeling in mice was associated with improved myocardial energy metabolism [36].

6.4.3 *Obesity and Diabetes*

Chronic inflammatory disease initiated in adipose tissue might lead to obesity-related insulin resistance and may contribute to an increased risk of diabetes [132]. It might be assumed that anti-inflammatory phytochemicals may protect against both diseases. Thus, I3C was also proposed as a potential preventive agent against obesity and metabolic disorders. In this regard, I3C treatment in diet-induced obesity (DIO) mice model decreased body weight and fat accumulation and infiltrated macrophages in epididymal adipose tissue. These effects were associated with improved glucose tolerance and with modulated expression of adipokines and lipogenic-associated gene products, including acetyl coenzyme A carboxylase and peroxisome proliferator-activated receptor-γ (PPARγ) [19]. The reduced level of inflammatory biomarkers was also confirmed in co-culture of adipocytes with macrophages treated with I3C [19].

I3C was also capable to normalize tissue expression of genes related to thermogenesis upregulated by high-fat diet, namely uncoupling proteins 1 and 3, PPARα, PPARγ coactivator 1α [26]. The observed improvement of adipogenesis by I3C could be due to activation of sirtuine SIRT1 [27]. These findings suggest that I3C has a potential benefit in preventing obesity and metabolic disorders, and the action for I3C in vivo may involve multiple mechanisms including decreased adipogenesis and inflammation, along with activated thermogenesis.

Little is known about the possible modulation of different types of diabetes by I3C. Nevertheless, in recent studies with the genetically modified mice (C57BL/6J mice) that closely simulated the metabolic abnormalities of the human disease after the administration of high-fat diet, both I3C and DIM showed a positive modulation of glucose, insulin, hemoglobin and glycated hemoglobin levels. In the same time a decreased levels of different mediators of oxidative stress were noticed, including

thiobarbituric acid reactive substances (TBARS), lipid hydroperoxides (LOOH) and conjugated dienes. Simultaneously, in this diabetic mouse model increased levels of antioxidant enzymes and small molecules (SOD, CAT, GPx, vitamin C, vitamin E, GSH) were demonstrated. Interestingly, the antioxidant action was comparable to that of metformin, a standard drug in diabetes 2 treatment [60].

6.5 Biological Activities of Indole-3-Carbinol in Animal Models

Animal models played a crucial role in discovering the cancer chemopreventive activity of cruciferous plants. Initially rodent chemical carcinogenesis models were used to assess anti-carcinogenic activity of minor dietary components, including I3C. Currently genetically modified animals, mentioned in the previous sections, allow to assess detailed mechanisms of their biological activity.

In the very first experiments of Wattenberg and his co-workers, benzo[a]pyrene induced model of lung and forestomach cancer in mice and dimethylbenz[a]anthracene induced breast neoplasia in rats were used. In these models I3C when given prior to carcinogen inhibited the formation of tumors [122]. This effect was linked to modulation of phase I enzymatic systems, namely cytochrome P450 dependent monooxygenases, involved in carcinogens activation [123, 124]. Later, several studies using animals carcinogenesis model of liver, colon, and tongue confirmed the anti-initiating activity of I3C. However, these studies have also provided evidence for promotional activity of I3C. For example, whereas I3C pretreatment and co-treatment with liver carcinogen aflatoxin B_1 (AFB_1) strongly inhibited AFB_1 initiated hepatocarcinogenesis, posttreatment with I3C was strongly promotional [35].

The modulation of cytochromes P450 is also linked with potential protection against breast cancer. On the other hand, the same mechanism is probably responsible for uterine-induced cancer via upregulation of CYP1B1 and increased precancerous 4-hydroxyestrogen concentration [133]. The increase of carcinogenic 4-hydroxyestrogen following oral administration of I3C were documented also by the other studies [56]; reviewed in [15]. These observations led to conclusion that DIM showing higher bioavailability and reducing 4-hydroxyestrogen production should be recommended as an alternative to I3C in potential chemopreventive supplementation [15]. More recent studies have confirmed this suggestion that DIM was more effective in prevention prostate cancer in the transgenic adenocarcinoma mouse prostate (TRAMP) mice model then I3C [25].

Nevertheless, the more recent studies using “traditional” mouse models or transgenic animals documented that I3C has been responsible for a decrease of incidences of carcinogen-induced lung cancer [64, 88, 102], cervical cancer in HPV gene transgenic mice [61], and UV-induced skin cancer [29].

Moreover, it was shown that in rats bearing the 13762 mammary carcinoma, addition of I3C to the diet for 6 days prior to antitumor drug ET-743 (trabectidin) administration almost completely abolished manifestations of hepatotoxicity [41]. These observation further supports the concept that I3C or DIM protecting against specific cancer may be used in adjuvant therapy to overcome side-effects of conventional therapy.

Specific rodent models like mouse carotid artery injury were developed and used to assess I3C or DIM protection against cardiovascular diseases, obesity, or diabetes. As it was described in previous section, generally the results of these studies suggest that I3C has a potential benefit in preventing obesity and metabolic disorders, and the action of I3C in vivo may involve multiple mechanisms including decreased adipogenesis and inflammation, along with activated thermogenesis.

6.6 Biological Activities of Indole-3-Carbinol in Humans

Promising results of the most studies obtained in human cancer cell lines and in animal models prompted the clinical trials dietary intervention studies to evaluate the effect of I3C or DIM in risk group of patients or/and volunteers. A major focus of these trials has been on modulation of hormones metabolism. The urinary estrogen metabolite ratio of 2-hydroxyesterone to 16α-hydroxyestrone was used in most of the trials as the surrogate endpoint biomarker.

The validity of this endpoint biomarker was confirmed in the early randomized clinical trials [14] in which 20 healthy subjects received 400 mg/day of I3C for 3 months. In most of the enrolled subjects I3C increased the 2-hydroxyestrone to estriol (a precursor of 16α-hydroxyestrone) ratio in sustained manner without detectible side-effects, although some individuals were resistant to such change. In another trial women at increased risk for breast cancer were administered with different doses (range 50–400 mg/day) of I3C for 4 weeks. The results of this study suggested that the minimum effective dose schedule of 300 mg/day is optimal for breast cancer prevention, although should be confirmed by long-term breast cancer prevention trial [128].

In subsequent studies by Bradlow group [91] urine samples were collected from healthy subjects before and after oral ingestion of 6–7 mg/kg per day for 1 week (7 men) or 2 months (10 women). Analysis of 13 estrogen profiles supported the hypothesis that I3C induces estrogen 2-hydroxylation resulting in decreased concentrations of metabolites known to activate the estrogen receptor and suggested that I3C may have chemopreventive activity against breast cancer in humans. Later, phase I trial with women with a high-risk breast cancer were enrolled, subjects ingested 400 mg I3C daily for 4 weeks followed by a 4 week period of 800 mg I3C daily [105]. The maximal ratio increase of the urinary 2-hydroxyestrone to 16α-hydroxyestrone was observed with the 400 mg daily dose of I3C, with no further increase found at 800 mg daily. Beside confirmation of the optimal dose of

I3C, these studies showed the induction of CYP1A2 which was mirrored by increase of 2-hydroxyestrone to 16α-hydroxyestrone ratio, and GST.

Cumulative evidence on conversion of I3C to DIM in cell culture, peritoneal and oral use as well as substantial direct activity seen with DIM led to conclusion that there is no longer the case for considering I3C to be directly active, and rather DIM should be considered as a chemopreventive compound of choice [15]. A pilot study on the effect of BR-DIM on urinary hormone metabolites in postmenopausal women with a history of early-stage breast cancer showed a significant increase in levels of 2-hydroxyestrone as result of treatment with only 108 mg DIM/day for 30 days, however, nonsignificant increase (1.46–2.14) of 2-hydroxyestrone to 16α - hydroxyestrone was noted [33]. In another study cohorts of 3–6 patients castrate-resistant, non-metastatic prostate cancer received escalating oral doses twice daily of BR-DIM 75 mg, then 150, 225, and 300 mg. Based on the results of this trial 225 mg BR-DIM dose twice daily was recommended for phase II trial. However, modest efficacy of DIM was demonstrated [53].

Cervical intraepithelial neoplasia (CIN) is a precancerous lesion of cervix. When patients with biopsy proven CIN grade II or III were treated orally with 200, or 400 mg/day of I3C for 12 weeks 50 % of them had complete regression based on their 12-week biopsy. Moreover, 2-hydroxyestrone to 16α-hydroxyestrone ratio have changed in a dose-dependent manner [11]. The significant improvement in confirmed CIN I or II grade was also observed as a result of oral treatment with 2 mg/kg/day of DIM for 12 weeks. Moreover, at median follow-up of 6 months there was no statistically significant difference in any of the measured outcome between the DIM and placebo group [40].

Since the incidence of thyroid cancer is 4–5 times higher in women than in men, estrogens were suggested to contribute the pathogenesis of thyroid proliferative disease (TPD). In limited (7 patients) phase I clinical trial patients with TPD were administered with 300 mg of DIM per day for 14 days. DIM was detectable in thyroid tissue, and the ratio of 2-hydroxyestrone to 16α-hydroxyestrone was increased. These results suggested that DIM can manifest the anti-estrogenic activity in situ to modulate TPD [104].

Although major focus of cancer prevention clinical trials of I3C or DIM has been concentrated on chemoprevention of hormone-dependent cancers, there were also clinical trials performed in order to evaluate indoles effect on pulmonary cancers. In this regard in phase I clinical trial patients with recurrent respiratory papillomatosis (RRP) were treated orally with I3C and had minimum follow-up of 8 months. Thirty-three percent of the study patients had a cession of their papilloma growth and had not required surgery since the start of the study [107]. Subsequent long-term clinical trial performed by the same research group confirmed the preliminary observation indicating that I3C may be a treatment option for RRP [108]. The case of successful use of intralesional and intravenous cidofovir in association with I3C in 8-year-old girl with pulmonary papillomatosis was also reported [38].

As it was mentioned in the previous sections of this chapter, recently a large amount of evidence has demonstrated that epigenetic alterations, such as DNA

methylation, histone modifications, and non-coding miRNAs consistently contribute to carcinogenesis, and constituents in the diet, including dietary glucosinolate derivatives, have the potential to alter a number of these epigenetic events [46]. Different studies on cancer also have shown that miRNAs interact with genes in many different cellular pathways, displaying a differential gene expression profile between normal and tumor tissues and between tumor types [17]. Interestingly, interventions including BR-DIM in prostate cancer patients prior to radical prostatectomy showed re-expression of miR-34a, which was consistent with decreased expression of androgen receptor, prostate specific antigen (PSA), and Notch-1 in tissue specimens [70]. These results suggest that BR-DIM could be useful for the inactivation of androgen receptor, critically important during the development and progression of prostate cancer and thus its treatment.

Thus far, seven clinical studies have been registered using I3C and twelve using DIM (www.clinicaltrials.gov; accessed Dec 26, 2015). Four studies registered for I3C treatment have been completed for patients with prostate and breast cancers and one dietary intervention for healthy participants targeting unspecified adult solid tumors. One trial aiming at I3C effects on estrogen metabolism in obese volunteers had to be terminated because of slow accrual in the high BMI group. Among twelve studies registered for DIM, six have been completed for patients with prostate, breast, and cervical cancers as well as healthy volunteers. Trial aiming at new therapy of laryngeal papilloma in children was terminated because of lack of sufficient enrollment. Although the results of these trials have not been published yet, they assure the further extensive prospective studies on chemopreventive and/or chemotherapeutic potential of I3C and its condensation product.

6.7 Summary and Conclusions

It is well known that in populations which consume higher amounts of cruciferous vegetables lower incidence rate of cancer occurs or improved biochemical parameters, such as decreased oxidative stress are noticed [46, 117, 118, 121]. These effects are in part due to the biological activity of I3C and its condensation products, particularly DIM.

A wide range of cellular pathways are regulated by both indoles. Thus, many additional targets for indoles could be identified in the future using in vitro cell cultures and in vivo transgenic animal models and explain a unique anti-inflammatory and endocrine modulating activity of I3C. Although most of current data support the role of I3C and DIM in prevention of hormone-dependent cancers, it seems that their application in prevention of the other cancer as well as cardiovascular diseases, obesity, and diabetes reduction is also possible.

Experimental in vitro and in vivo studies and clinical trials performed so far, showed that I3C is a rather safe dietary supplement. However, since the long-term effects of I3C supplementation in humans are still not clear and due to some contradictory effects of I3C in animal models, the general use of I3C and DIM

supplements should be restricted until potential risks and benefits are better characterized. Taking into consideration higher activity of DIM, particularly in BR form, in comparison with I3C in term of potency and time required to obtain the effect, this I3C dimer might be a better alternative as chemopreventive supplement. Important aspect of possible clinical application of both indoles is their drug and radio-sensitization. Emerging new technologies allowing deeper inside in the mechanism of these glucosinolate derivatives activity should help to better explore this aspect.

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