

# International Research Conference on Food, Nutrition, and Cancer

## Indole-3-Carbinol and Prostate Cancer<sup>1,2</sup>

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**ABSTRACT** Epidemiological and dietary studies have revealed an association between high dietary intake of cruciferous vegetables and decreased prostate cancer risk. Our studies have shown that indole-3-carbinol (I3C), a common phytochemical in cruciferous vegetables, and its in vivo dimeric product 3,3'-diindolylmethane (DIM) upregulate the expression of phase I and phase II enzymes, suggesting increased capacity for detoxification and inhibition of carcinogens. Studies from our laboratory and others have found that I3C can induce G1 cell-cycle arrest and apoptosis in prostate cancer cells. In addition, we found, by microarray gene expression profiling, that I3C and DIM regulate many genes that are important for the control of cell cycle, cell proliferation, signal transduction, and other cellular processes, suggesting the pleiotropic effects of I3C and DIM on prostate cancer cells. We recently found that I3C functions as an inhibitor of Akt and nuclear factor  $\kappa$ B (NF- $\kappa$ B), which play important roles in cell survival and which are believed to be potential targets in cancer therapy. Studies have already shown that the inactivation of Akt and NF- $\kappa$ B is responsible for chemosensitization of chemoresistant cancer cells. Because there is no effective treatment strategy for hormone-dependent and, most importantly, hormone-independent and metastatic prostate cancer, our strategies to sensitize prostate cancer cells to a chemotherapeutic agent by I3C and DIM is a novel breakthrough that could be used for devising novel therapies for prostate cancer. In conclusion, the results from our laboratory and from others provide ample evidence for the benefit of I3C and DIM for the prevention and the treatment of prostate cancer. *J. Nutr.* 134: 3493S–3498S, 2004.

**KEY WORDS:** • I3C • prostate cancer • prevention • treatment

Prostate cancer is one of the most common cancers in men and is the second leading cause of male cancer death in the United States (1). An estimated 220,900 new cases and 28,900 deaths from prostate cancer cells occurred in 2003 (1). However, men in Asia have a much lower incidence and mortality of prostate cancer than do men in North America and Europe (2,3). The differences in the cancer incidence among ethnic groups are believed to be due to different lifestyles and environmental factors. Asians who emigrated from their native countries to the United States and adopted Western lifestyles typically experienced an increasing incidence of hormone-related cancers (4,5), suggesting that the diet in

their native countries may have a role in protecting against hormone-related cancers. It has been estimated that more than two-thirds of human cancers can be prevented by modification of lifestyle, including dietary modification. The consumption of fruits, soybeans, and vegetables has been associated with reduced risk of several types of cancers (6–8). Epidemiological and dietary studies have shown an association between high dietary intake of vegetables and decreased prostate cancer risk (7,8). Indole-3-carbinol (I3C),<sup>4</sup> a phytochemical common in cruciferous vegetables, inhibits carcinogenesis in animal experiments and growth of various cancer cells in culture (9–11). I3C and its in vivo dimeric product 3,3'-diindolylmethane (DIM) have received much attention in recent years as cancer preventive agents.

I3C is produced from naturally occurring glucosinolates contained in a wide variety of plants, including members of the family Cruciferae and particularly members of the genus *Brassica*. I3C is biologically active, and it is easily converted in

<sup>1</sup> Published in a supplement to *The Journal of Nutrition*. Presented as part of the International Research Conference on Food, Nutrition, and Cancer held in Washington, DC, July 15–16, 2004. This conference was organized by the American Institute for Cancer Research and the World Cancer Research Fund International and sponsored by BASF Aktiengesellschaft; Campbell Soup Company; The Cranberry Institute; Danisco USA Inc.; DSM Nutritional Products, Inc.; Hill's Pet Nutrition, Inc.; Kellogg Company; National Fisheries Institute; The Solae Company; and United Soybean Board. An educational grant was provided by The Mushroom Council. Guest editors for this symposium were Helen A. Norman, Vay Liang W. Go, and Ritva R. Butrum.

<sup>2</sup> This work was partly funded by grants from the Department of Defense (DOD Prostate Cancer Research Program DAMD17-03-1-0042 awarded to F.H.S.), NIH (1R01CA108535-01 awarded to F.H.S.), and Puschelberg Foundation (F.H.S.).

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<sup>4</sup> Abbreviations used: ATF, activating transcription factor; CBFB, core binding factor beta; CDK, cyclin-dependent kinases; CDKI, CDK inhibitor; DIM, 3,3'-diindolylmethane; EGF, epidermal growth factor; I3C, indole-3-carbinol; IKK, I $\kappa$ B kinase; MIG, mitogen inducible gene; MAPK, mitogen-activated protein kinase; NF- $\kappa$ B, Nuclear factor  $\kappa$ B; NF-YC, nuclear factor YC; PARP, poly(ADP-ribose) polymerase; PI3K, phosphatidylinositol-3 kinase; PDK1, phosphoinositide-dependent protein kinase 1; ST16, suppressor of tumorigenicity 16; TFDP, transcription factor Dp.

vivo to DIM, which is also biologically active (12). Vegetables of the genus *Brassica* contribute most to our intake of glucosinolates and include all kinds of cabbages, broccoli, cauliflower, and Brussels sprouts. All glucosinolates share a common basic skeleton containing a glucose group, a side chain, and a sulfonated oxime moiety but differ in the side chain. Glucosinolates with an indole side chain form indoles. The most prevalent glucosinolate with an indole side chain is glucobrassicin, which is predominant in brassica vegetables. When hydrolysis occurs, glucobrassicin forms an unstable isothiocyanate that degrades to I3C (13). Under the acidic conditions of the stomach, I3C undergoes extensive and rapid self-condensation reactions to form DIM (13,14) (Fig. 1).

Because of its anticarcinogenic effects in experimental animals (11,15,16) and humans (17–19), I3C has received special attention as a possible chemopreventive agent (13). I3C has also been found to inhibit the growth of various cancer cells (20–23) and possibly to inhibit breast cancer invasion and migration (24,25). Because of its pleiotropic effects, studies on I3C as a cancer chemopreventive agent have increased significantly in recent years. However, the molecular mechanisms by which I3C exerts its tumor suppressive effects on prostate cancers have not been fully elucidated. In this article, we summarized our data regarding the inhibitory effects of I3C and DIM on prostate cancer cells and provided a comprehensive view on the molecular mechanisms of cancer prevention and treatment by I3C and DIM.

### I3C inhibits cell growth and induces cell-cycle arrest in prostate cancer cells

I3C inhibits the growth of breast, prostate, colon, and cervical cancer cells (9,20,26–28). Data from our laboratory showed that I3C and DIM inhibited the growth of PC3 prostate cancer cells (29–31). The inhibition of cell growth was

found to be dose and time dependent. I3C at 60  $\mu\text{mol/L}$  and DIM at 30  $\mu\text{mol/L}$  significantly inhibited PC3 cell growth. This growth inhibition could be due to cell-cycle arrest, which ultimately results in the cessation of cell proliferation. By flow cytometry analysis, we found that I3C induced G1 cell-cycle arrest in PC3 prostate cancer cells (29), in accord with the report showing that I3C induces G1 cell-cycle arrest in breast cancer cells (32).

Because the induction of cell-cycle arrest in prostate cancer cells by I3C could be mediated via regulation of the expression of genes involved in the control of the cell cycle, we further examined the status of cyclins, cyclin-dependent kinases (CDK), and CDK inhibitors (CDKI) in I3C-treated PC3 prostate cancer cells. By Western blot analysis, we found that I3C downregulated the expression of CDK6 and upregulated the expression of p21<sup>WAF1</sup> and p27<sup>KIP1</sup> in a dose-dependent manner (29). We immunoprecipitated cyclin D1 and cyclin E complexes, and detected CDK6, p21<sup>WAF1</sup>, and p27<sup>KIP1</sup> in the complexes. The results showed that I3C decreased CDK6 binding to cyclin D1 and increased p21<sup>WAF1</sup> and p27<sup>KIP1</sup> binding to cyclin D1 and cyclin E complexes (29), suggesting the inhibition of cell-cycle progression. We also found that I3C inhibited the CDK6 kinase activity (29), which plays important roles in G1 regulation. These findings were consistent with results showing cell-growth inhibition and cell-cycle arrest induced by I3C, suggesting that I3C inhibits the growth of prostate cancer cells through regulation of genes related to the control of cell proliferation and the cell cycle. Reports from other laboratories also showed that I3C inhibited the expression of CDK6 and induced G1 arrest in breast cancer cells (32,33). The upregulation of p21<sup>WAF1</sup> and p27<sup>KIP1</sup> and the downregulation of CDK6 may be one of the molecular mechanisms by which I3C inhibits prostate cancer cell growth and induces cell-cycle arrest (Fig. 2).

### I3C induces apoptosis in prostate cancer cells

I3C induces apoptotic cell death in breast cancer cells (27,34). We conducted the DNA ladder assay, the poly(ADP-ribose) polymerase (PARP) assay, and the flow cytometric analysis with 7-amino actinomycin D (7AAD) staining to detect apoptosis in PC3 prostate cancer cells treated with I3C. By using these different techniques, we found that I3C at 60–100  $\mu\text{mol/L}$  induced apoptosis in PC3 prostate cancer cells (29). DNA ladder formation and PARP cleavage were observed in prostate cancer cells treated with I3C for 48 h. Flow cytometry analysis revealed that the number of apoptotic cells increased up to 58.49% with I3C treatment at 100  $\mu\text{mol/L}$  for 48 h and reached up to 80% with longer I3C treatment (30). These results clearly demonstrated that I3C induces apoptosis in prostate cancer cells, which is consistent with studies in breast cancer cells (27,34).

To find the molecular mechanisms by which I3C induces apoptosis, we investigated the alteration of protein expression of genes related to the apoptotic pathway. Bax, Bcl-2, and Bcl<sub>XL</sub> play important roles in determining whether cells will undergo apoptosis (35,36). Bax promotes apoptotic cell death, whereas Bcl-2 and Bcl<sub>XL</sub> protect cells from apoptosis. The ratio of Bax to Bcl-2 rather than Bcl-2 alone was reported to be an important factor for determining whether cells survive or undergo apoptosis (37). By Western blot analysis, we found decreases in Bcl-2 and Bcl<sub>XL</sub> protein expression in PC3 prostate cancer cells treated with I3C at 60  $\mu\text{mol/L}$  for 48 h and longer (29,30). However, the expression of Bax was upregulated after I3C treatment for 24 h. The ratio of Bax to Bcl-2 was significantly increased after 24 h of I3C treatment corre-

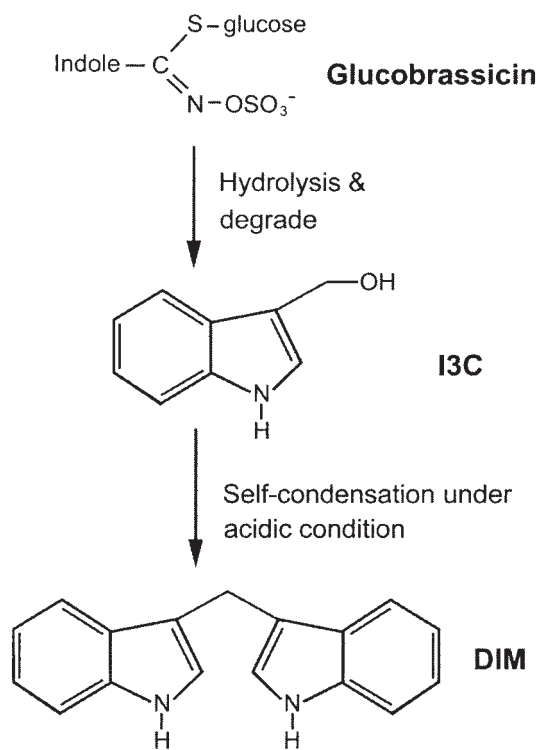
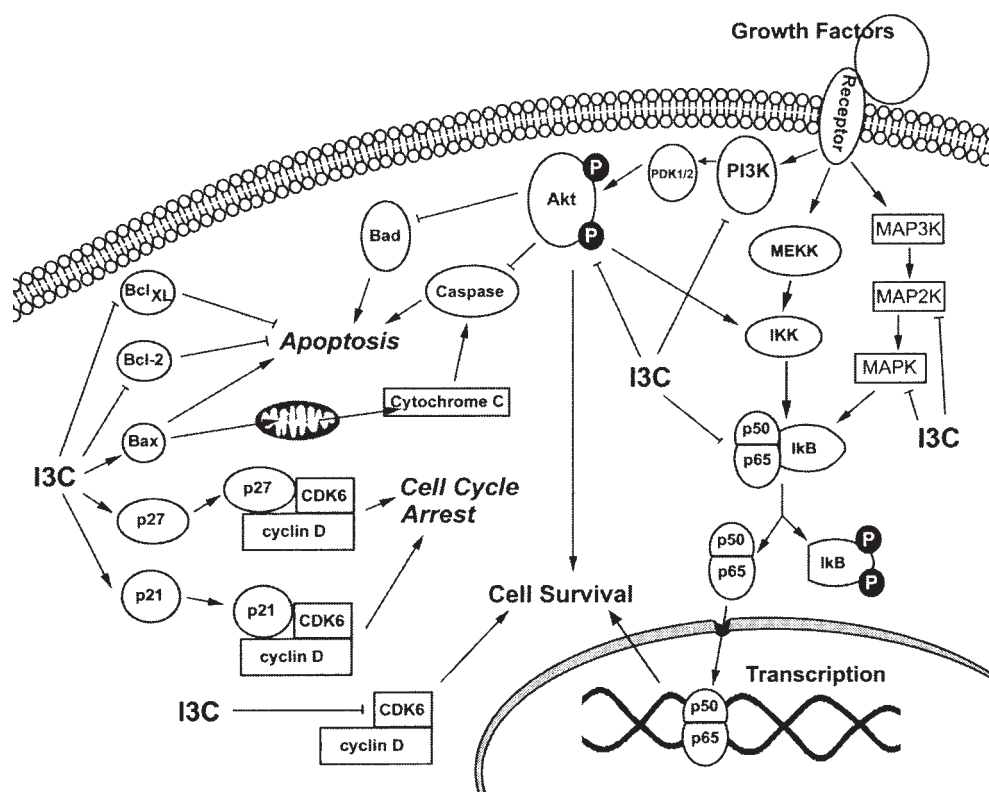


FIGURE 1 Molecular structure and metabolism of I3C and DIM.



**FIGURE 2** The effects of I3C on cell cycle, apoptosis, NF- $\kappa$ B, Akt, and MAPK pathways.

sponding to a significant increase in apoptotic cells after I3C treatment for 48 h. Similar results were also observed in I3C-treated breast cancer cells in our laboratory (38,39). These results suggest that upregulation of Bax and downregulation of Bcl-2 and Bcl<sub>XL</sub> may be one molecular mechanism by which I3C induces apoptosis (Fig. 2).

Bax translocation from cytosol into mitochondria also plays important roles in the induction of apoptosis (40). The translocation of Bax from cytosol into mitochondria targets the mitochondrial intermembrane contact sites, causing the mitochondrial permeability transition, loss of mitochondrial potential, release of cytochrome c, subsequent activation of caspases, and DNA fragmentation, resulting in apoptosis (40,41). To further explore the mechanism of I3C-induced apoptosis, we investigated Bax localization, mitochondrial potential, and cytochrome c in both MCF10A nontumorigenic cells and MCF10CA1a cancer cells treated with I3C (42). By using immunostaining and confocal imaging techniques, we observed the translocation of Bax from the cytosol into the mitochondria in both cell lines treated with I3C. However, the loss of mitochondrial potential and the release of cytochrome c induced by I3C were only observed in MCF10CA1a breast cancer cells. No such effects or significant apoptosis were observed in MCF10A nontumorigenic cells, suggesting that I3C-induced loss of mitochondrial potential is a more important event for the release of cytochrome c and induction of apoptosis in cancer cells. We also observed that DIM selectively induces apoptosis in PC3 prostate cancer cells but not in CRL-2221 nontumorigenic cells, suggesting that I3C and DIM may be the ideal agents for the prevention and the treatment of prostate and other cancers.

#### **I3C inhibits the nuclear factor $\kappa$ B pathway in prostate cancer cells**

The nuclear factor  $\kappa$ B (NF- $\kappa$ B) pathway plays an important role in many physiological processes in cellular signaling (43–

45). In human cells, NF- $\kappa$ B is sequestered in the cytoplasm through tight association with its inhibitory protein, I $\kappa$ B. The activation of NF- $\kappa$ B occurs through site-specific phosphorylation of I $\kappa$ B by I $\kappa$ B kinase (IKK). I $\kappa$ B is subsequently degraded by the 26S proteasome. NF- $\kappa$ B becomes free from I $\kappa$ B and translocates into the nucleus for binding to NF- $\kappa$ B-specific DNA-binding sites, regulating target gene transcription. IKK- $\alpha$  also phosphorylates histone H3 and regulates the activation of NF- $\kappa$ B-directed gene expression. NF- $\kappa$ B can be activated by many types of stimulators (43–46), including carcinogens [TNF, 9,10-dimethyl-1,2-benzanthracene (DMBA), cigarette smoke condensate, etc.], tumor promoters (phorbol myristate acetate, etc.), stress (pH, hypoxia, heavy metals, hydrogen peroxide, etc.), endotoxin (LPS, etc.), apoptosis inducers (chemotherapeutic agents, cytokines, etc.), infection (bacterial, viral, etc.), and cytokines [IL-1, IL-17, IL-18, epidermal growth factor (EGF), etc.]. Activated NF- $\kappa$ B controls the expression of genes that are involved in controlling cell proliferation, differentiation, apoptosis, inflammation, stress response, angiogenesis, tumor promotion and metastasis, and other cellular and physiological processes (43–45). Because of its critical effects on tumor development and progression, NF- $\kappa$ B has been described as a major culprit and a therapeutic target in cancer (43,46,47). Inhibition of NF- $\kappa$ B activation is generally believed to suppress tumorigenesis and the progression of tumors.

We used an electrophoretic mobility shift assay to investigate whether I3C treatment inhibits NF- $\kappa$ B DNA binding activity in PC3 prostate cancer cells (29). PC3 cells were treated with I3C at 60  $\mu$ mol/L for 48 h or TNF- $\alpha$  at 50  $\mu$ g/L for 15 min. Nuclear extracts were harvested from samples, incubated in binding buffer with <sup>32</sup>P-labeled NF- $\kappa$ B consensus oligonucleotide, and subjected to 8% nondenatured polyacrylamide gel. Autoradiography of the dried gel showed that TNF- $\alpha$  treatment stimulated NF- $\kappa$ B activation as expected; however, I3C at 60  $\mu$ mol/L significantly inhibited NF- $\kappa$ B

DNA binding, corresponding to inhibition of cell proliferation and induction of apoptosis by I3C in PC3 prostate cancer cells. These results suggest that inhibition of NF- $\kappa$ B pathway by I3C may be another molecular mechanism by which I3C inhibits cell proliferation and induces apoptosis (Fig. 2).

### I3C inhibits Akt pathway in prostate cancer cells

The Akt signaling pathway is another important signal transduction pathway in human cells and plays a critical role in controlling the balance between cell survival and apoptosis (48–50). This pathway can be activated by various growth and survival factors, such as EGF, platelet-derived growth factor, insulin, etc., through activation of phosphatidylinositol-3 kinase (PI3K) (48). PI3K activation leads to the production of phosphatidylinositol-3,4,5-trisphosphate, which interacts with the Akt PH domain. The interaction subsequently causes Akt conformational changes, resulting in exposure of 2 main phosphorylation sites in Akt. Akt is then activated by phosphorylation at Thr308 by phosphoinositide-dependent protein kinase 1 (PDK1) or at Ser473 by PDK2 (48). Activated Akt promotes cell survival by inhibiting apoptosis through its ability to phosphorylate and to inactivate several targets, including Bad, Forkhead transcription factors, and caspase-9 (50), all of which are involved in the apoptotic pathway. More importantly, Akt also activates the NF- $\kappa$ B pathway through phosphorylation of molecules in the NF- $\kappa$ B pathway (51), suggesting its role in promoting cell survival. Because of its importance in cell survival, Akt is also believed to be a target in cancer therapy.

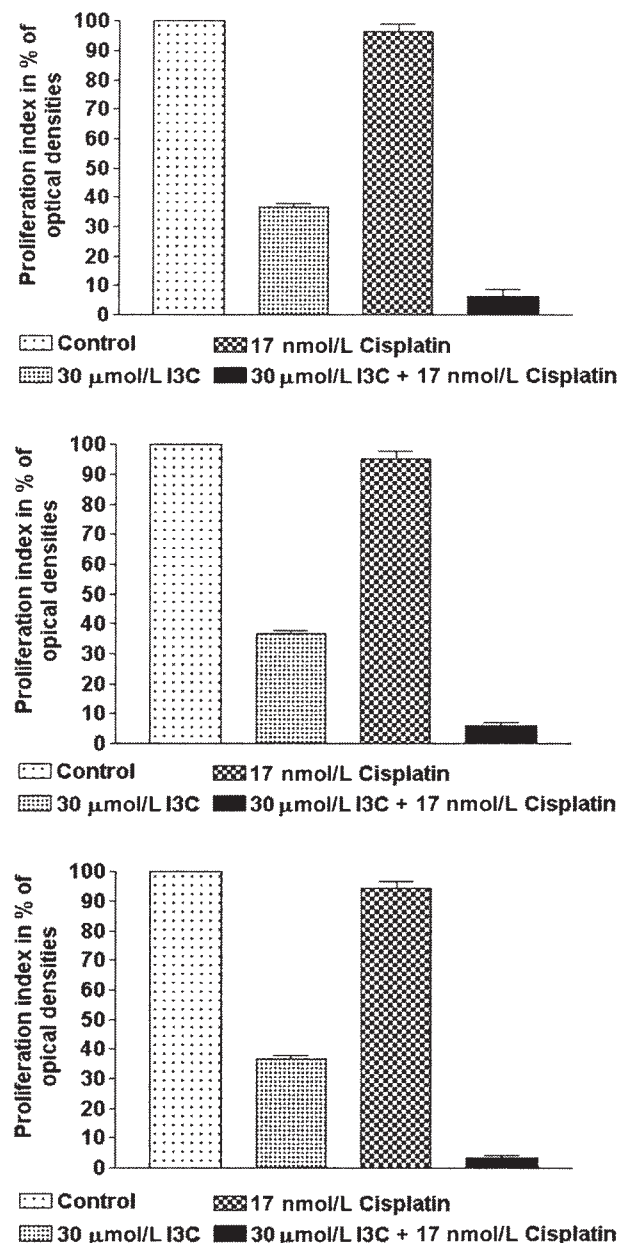
To investigate whether I3C-induced cell-growth inhibition and induction of apoptosis occurs through the Akt pathway, we examined Akt status in PC3 cells treated with I3C at 30–100  $\mu$ mol/L by Western blot analysis (30). We did not find any change in the level of total Akt protein expression after I3C treatment. However, we observed a decrease in the phosphorylated Akt protein at Ser473 and Thr308 in the I3C-treated PC3 cells compared with control cells, suggesting inactivation of Akt kinase after I3C treatment. Immunoprecipitation and Akt kinase assay showed that I3C downregulated Akt kinase activity in PC3 cells, consistent with the data from Western blot analysis. We also examined the Akt status in the PC3 cells pretreated with I3C followed by EGF stimulation. We found that EGF upregulated Akt kinase activity; however, I3C pretreatment abrogated EGF-induced activation of Akt. These data clearly demonstrated that I3C inhibits Akt activation both with and without stimulation. From the gene expression profiles of I3C-treated PC3 cells, we also found downregulation of PI3K expression (31), corresponding to our results, showing I3C-induced inactivation of Akt kinase activity. Inhibition of Akt activity with downregulation of Bcl-2 and Bcl<sub>XL</sub> may result in the inhibition of survival signals and may also induce apoptotic signals. Thus, the inhibition of Akt pathways by I3C may be another molecular mechanism by which I3C induces apoptosis in prostate cancer cells (Fig. 2).

We also showed that forced overexpression of Akt in prostate cancer cells by Akt gene transfection leads to the activation of NF- $\kappa$ B (52). The activation of Akt and NF- $\kappa$ B is believed to be responsible for the resistance to chemotherapeutic agents, which is the major cause for treatment failure in cancer chemotherapy. Inhibition of NF- $\kappa$ B or Akt can potentiate the anticancer effect of chemotherapeutic agents (53,54). We found that I3C inhibited the activation of Akt and NF- $\kappa$ B and induced apoptosis, suggesting that I3C can sensitize cancer cells to apoptosis induced by chemotherapeutic agents. By cell growth inhibition assay, we found that combination treat-

ment with I3C and cisplatin significantly inhibited the growth of PC-3 prostate cancer cells compared with monotherapy (Fig. 3). These results suggest that I3C and DIM can potentiate the anticancer effect of chemotherapeutic agents through inactivation of Akt and NF- $\kappa$ B.

### I3C and DIM regulate gene expression profiles in prostate cancer cells

To explore the precise molecular mechanisms by which I3C and DIM exert their pleiotropic effects on prostate cancer cells, we used the high-throughput gene chip, which contains 22,215 known genes, to determine the alternation of gene expression profiles of PC3 prostate cancer cells exposed to I3C or DIM (31). By microarray analysis, we found that both I3C and DIM regulated many genes related to cell proliferation, cell-cycle control, apoptosis, signal transduction, oncogenesis,



**FIGURE 3** Growth inhibition of PC-3 prostate cancer cells exposed to I3C and cisplatin for 72 h (repeated 3 times).

and transcription regulation, suggesting that I3C and DIM alter biological processes and molecular functions in PC3 cells through a variety of cell-signaling pathways. The global gene expression profiles of prostate cancer cells after I3C or DIM treatment provided important information for further investigation of the molecular mechanisms by which I3C and DIM inhibit prostate cancer cells.

EGF receptor, transforming growth factor- $\beta$ , and fibroblast growth factor play important roles in promoting cell growth and angiogenesis. From microarray analysis, we found that I3C and DIM downregulated their expression, corresponding to the growth inhibitory effects of I3C and DIM. Cyclin E, Bcl-2, activating transcription factor (ATF), and mitogen inducible gene (MIG) promote cell-cycle progression and inhibit apoptosis (55,56). We found that I3C and DIM inhibited the expression of cyclin E2, ATF5, MIG-2, and Bcl-2, and induced the expression of p57<sup>KIP2</sup>, suggesting the effects of I3C and DIM on the induction of cell-cycle arrest and apoptosis.

From microarray analysis, we also found that I3C and DIM also regulated other cell-signal transduction pathways, e.g., the mitogen-activated protein kinase (MAPK) pathway, which consists of a 3-tiered kinase core where a MAP3K activates a MAP2K that activates a MAPK (57,58). Because it promotes the activation of NF- $\kappa$ B and cell survival, MAPK has also been believed to be a target for cancer prevention and therapy (58). We observed a decrease in the expression of MAP2K3, MAP2K4, MAP4K3, and MAPK3 after I3C and DIM treatment, suggesting the inhibitory effects of I3C and DIM on the MAPK pathway, which could result in the inhibition of cancer-cell survival (Fig. 2).

Pol II transcription factors [transcription factor Dp (TFDP), nuclear factor YC (NF-YC), etc.] play important roles in transcription, cell-cycle progression, and oncogenesis (59,60). In addition, core binding factor beta (CBFB) and suppressor of tumorigenicity 16 (ST16) are also involved in the processes of oncogenesis. CBFB forms fusion proteins with other gene products and promotes oncogenesis, whereas ST16 suppresses the oncogenesis (59,60). Our results from gene expression profiles showed that I3C and DIM downregulated the expression of TFDP1, NF-YC, and CBFB, and upregulated ST16 expression, suggesting that I3C and DIM can inhibit transcription and oncogenesis in PC3 prostate cancer cells.

### Summary and perspective

In conclusion, I3C and DIM from the natural foods of the family Cruciferae exert anticancer effects mediated through the regulation of the cell cycle, cell proliferation, apoptosis, oncogenesis, transcription, and cell-signal transduction. The inactivation of Akt, NF- $\kappa$ B, MAPK, and Bcl-2 signaling pathways may be the molecular mechanisms by which I3C and DIM inhibit cell growth and induce apoptosis in prostate cancer cells. Because I3C functions as an inhibitor of NF- $\kappa$ B and Akt activation and induces apoptosis, I3C and DIM may also sensitize prostate cancer cells to apoptosis induced by chemotherapeutic agents. However, further in vitro and in vivo investigations, along with clinical trials, are needed to find whether I3C and DIM can fulfill their promise as chemopreventive agents, therapeutic agents, or both against human prostate cancer.

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